

COMPARATIVE OSTEOGENESIS OF EQUINE MESENCHYMAL STEM CELLS  
ISOLATED FROM BONE MARROW, ADIPOSE TISSUE AND SYNOVIUM

BY

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THESIS

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## **ABSTRACT**

The experiments in this study were carried out to define the osteogenic potential of equine MSCs derived from synovium, bone marrow, and adipose tissue maintained under the same culture conditions. Equine MSCs were isolated from synovium [SYN], bone marrow [BM] and adipose tissue [AD], expanded in monolayer culture for two passages, and then transferred to basal or standard osteogenic medium. At day 7 and 14, the phenotypic status of the cells was primarily evaluated by staining for mineralized matrix (Alizarin Red and Von Kossa) and the enzymatic activity of alkaline phosphatase (ALP). Other secondary indicators of osteogenic differentiation included in this study were mRNA levels of ALP and osteoblast-specific genes Runx2, Osterix, Osteonectin and Osteomodulin. Staining of the mineralized matrix of BM-MSCs cultured under osteogenic conditions, demonstrated a higher uptake of both Alizarin Red (AR) and Von Kossa (VK) stains, restricted to dense cellular aggregation (nodules). Stain up-take in SYN and AD cultures was also limited to infrequent sites of cellular aggregation but was less intense than in BM cultures. In osteogenic cultures, intense ALP activity was present within and immediately around the cell aggregates in BM cultures, whereas staining in the SYN and AD monolayers was far less intense and more diffusely distributed. BM-MSCs cultured under osteogenic conditions increased ALP mRNA expression and ALP activity. Osteogenic AD cultures also significantly increased ALP activity by day 14. In SYN cultures, basal ALP activity was comparatively low and, even under osteogenic conditions, there were negligible changes in ALP activity. These findings suggest that BM-MSCs are more able to undergo osteogenic differentiation than SYN- or AD-MSCs. Bone marrow-derived cells should be preferred for any application focused on bone regeneration.

To My Grandfather

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## CHAPTER 1

### INTRODUCTION

Adult mesenchymal stem cells (MSCs) are a promising resource for the treatment of equine orthopaedic disease because of their extensive proliferative ability and capacity for multilineage differentiation, including osteogenesis, chondrogenesis and tenogenesis.

Bone fractures are common not only in sport horses but also in pleasure and working horses. Bone tissue has the ability to self-repair; however, when fractures are complex and the bone column of the limb is unable to support the horse's body weight, the prognosis for successful repair is poor. In many cases, euthanasia becomes the most appropriate option to avoid animal suffering and excessive costs. The limitations of current methods for equine fracture repair, as well as other potential applications of equine MSCs for the induction of joint fusion and subchondral cyst treatment, has motivated the equine research community to investigate and identify the best sources of MSCs for bone repair.

Over the past several years, research in the Stewart lab has been focused on addressing the chondrogenic potential of synovially derived progenitor populations. The experiments presented in this thesis were focused on determining whether equine synovium-derived cell populations are capable of osteogenic differentiation and assessing the relative osteogenic potencies of equine MSCs isolated from synovium, bone marrow and adipose tissue under identical culture conditions. Determining the best source(s) of osteogenic equine MSCs will facilitate cell-based strategies to enhance bone formation and fracture repair in horses.

## CHAPTER 2

### LITERATURE REVIEW

#### **MESENCHYMAL STEM CELLS**

##### The MSC Niche

It is now accepted that mesenchymal stem cells (MSCs) reside, in very low numbers, in most tissues and body fluids (Friedenstein, Deriglasova et al. 1974; De Schauwer, Meyer et al. 2011). The term “niche” was first introduced in 1978 by Schofield (Schofield 1978) and refers to the microenvironment where adult stem cells reside and interact with their environment, including other non-stem cells, extracellular matrix and soluble molecules. Kolf and colleagues stated that all the above-mentioned components “act together to maintain the stem cells in their undifferentiated state” supporting tissue homeostasis by replacing cells lost due to normal cell replacement or injury, while also maintaining the critical stem cell reserve in the niche (Kolf, Cho et al. 2007).

Many examples have been cited in the literature describing MSC niches in a diverse range of tissues such as the bulge area of the hair follicle containing epithelial stem cells (Niemann and Watt 2002), the cells located above the Paneth cells in the base of the intestinal crypts of the small intestine (Booth and Potten 2000), and the hematopoietic stem cell niche located on endosteal surfaces of bone marrow cavity (Bianco 2011). All these described niches are located very close to vasculature, providing adequate support for the stem cell niche as well as providing a convenient conduit for mobilization and migration to distant sites.

## Sources of MSCs

### *Bone Marrow MSCs*

In horses, the common sites for bone marrow (BM) collection are the sternum and tuber coxae. BM collection from the sternum is performed in the midpoint of a sternebrae (Smith, Korda et al. 2003; Toupadakis, Wong et al. 2010). The 5<sup>th</sup> sternebrae, easily identified by ultrasonography, has been recommended as the safest site for collection, since this site is relatively easy to access and is caudal to the heart base (Kasashima, Ueno et al. 2011). The site for aspiration from the tuber coxae is localized from the ventral third of the iliac wing, approximately 4 cm axial to the tuber coxae (Toupadakis, Wong et al. 2010).

BM contains both hematopoietic and mesenchymal elements. The BM-MSCs have the ability to differentiate into multiple cell lineages including bone, cartilage, muscle, ligament, tendon and adipose tissue (Wakitani, Goto et al. 1994; Young, Butler et al. 1998; Pittenger, Mackay et al. 1999; Pittenger, Vanguri et al. 2002; De Bari, Dell'Accio et al. 2003). Progenitor cells with the ability to differentiate into osteoblast-like cells are also called 'osteoprogenitors' (Bellows, Aubin et al. 1986; Friedenstein, Chailakhyan et al. 1987; Owen 1988). The osteoprogenitor cells are located adjacent to the endosteal surface of the marrow space. In fact, explants cultures of human trabecular bone, as well as periosteum, release progenitor cells with the ability to differentiate toward the osteogenic, adipogenic and chondrogenic lineages *in vitro* (Noth, Osyczka et al. 2002; Wiesmann, Nazer et al. 2003), comparable to the activities of adult human BM-MSCs (Pittenger, Mackay et al. 1999). The BM extracellular matrix and stromal cell population collectively serve as structural and functional support for hematopoietic stem cells (Bianco, Riminucci et al. 2001). Hematopoietic stem cells (HSC), responsible for the generation



and replacement of all hematogenetic cell lineages, have been used for transplants of bone marrow in cancer patients following radio-isotopic marrow ablation for up to 40 years with successful outcomes (Thomas 2000). Within the marrow stromal cell population(s), MSCs with tri-lineage differentiation ability has been estimated to constitute 1 in 100,000 nucleated cells and are clearly different from HSCs or endothelial cells, based on their surface antigen expression profile (Pittenger, Mackay et al. 1999). In light of these analyses, and given the presence of different types of cells in BM aspirates and the very low percentage of MSCs within any aspirate, new methods to improve the purification of MSCs from marrow aspirates would be particularly beneficial for ongoing stem cell research and clinical applications.

#### *Adipose Tissue MSCs*

Adipose tissue (AD) is an attractive source of progenitor cells due to its easy accessibility and abundance (Zuk, Zhu et al. 2002; Kern, Eichler et al. 2006; Vidal, Kilroy et al. 2007; Colleoni, Bottani et al. 2009). In horses, the most common site for collection is just lateral to the base of the tail (Vidal, Kilroy et al. 2007; Toupadakis, Wong et al. 2010).

AD-MSCs are capable of differentiating into multiple cell lineages including adipogenic, chondrogenic, osteogenic and myogenic cell types (Jaiswal, Haynesworth et al. 1997; Halvorsen, Franklin et al. 2001; Zuk, Zhu et al. 2001; Mambelli, Santos et al. 2009). Im and co-workers reported that AD-MSCs are less effective in differentiating along the osteogenic lineage, compared with BM-MSCs (Im, Shin et al. 2005). However, samples from BM and AD were collected from different donors of different ages, potentially affecting the results. In fact, the *in vitro* proliferation and differentiation capacity of MSCs between donors is variable, the implications of which will be discussed later in this chapter. In contrast, DeUgarte et al found no

significant difference between BM-MSCs and AD-MSCs with regard to their osteogenic capacities (De Ugarte, Morizono et al. 2003). In Im's study, the cells used for differentiation experiments were third passage populations, whereas De Ugarte used cells at passage four. It is likely that the passage number influences the osteogenic potentials of AD-MSCs and/or BM-MSCs.

### *Synovial MSCs*

The synovial membrane (SYN) covers the inner surface of joint capsules and is composed of a few cell layers containing secretory synoviocytes, macrophage-like cells and a small number of synovial progenitors (SYN-MSCs) (Harvanova, Tothova et al. 2011). The synovium can be easily harvested during arthroscopic or arthrotomy procedures. A number of studies have been performed using human SYN-MSCs due to the availability of these cells from total joint replacement tissues (Djouad, Bony et al. 2005; Shirasawa, Sekiya et al. 2006).

SYN-MSCs are capable of multi-lineage differentiation (De Bari, Dell'Accio et al. 2001; Djouad, Bony et al. 2005; Harvanova, Tothova et al. 2011); however, SYN-MSCs' ability to differentiate toward the chondrogenic pathway is reportedly higher than MSCs from other sources (Sakaguchi, Sekiya et al. 2005; Shirasawa, Sekiya et al. 2006; Yoshimura, Muneta et al. 2007). Koga and colleagues performed an *in vivo* study in rabbits demonstrating that transplanted SYN-MSCs applied in a collagen gel and covered in periosteum, were able to repair a full-thickness articular cartilage defect with hyaline cartilage (Koga, Muneta et al. 2007). In agreement with the previous study, unpublished data from Stewart laboratory has shown that SYN-MSCs induced toward the chondrogenic lineage did not express endochondral pathway

markers (collagen type X and alkaline phosphatase). Instead, the non-hypertrophic phenotype of these cells was similar to articular cartilage.

### Isolation of MSCs

In 1968, Friedenstein and co-workers first demonstrated the ability of the stromal portion of the BM to adhere to tissue culture plastic. In the same study, it was also recognized that the hematopoietic fraction of the BM lacks this capacity for adhesion (Friedenstein, Petrakova et al. 1968). However, other cell types present in marrow aspirates, such as macrophages, endothelial cells, lymphocytes and smooth muscle cells, can also adhere to plastic and contaminate BM preparations (Deans and Moseley 2000). In order to separate the MSC fraction from other cells type, various BM-MSC isolation protocols has been developed including the Classic, Percoll/Ficoll and Red Blood cell (RBC) lysis buffer strategies. The Classic protocol is based on the ability of MSCs to adhere to tissue culture plastic, as demonstrated by Friedenstein. Following BM collection, the aspirate is plated on culture plastic dishes and allowed to attach for 5 days. Then, the non-adherent cells are removed when the culture medium is changed (Caplan 1991; Haynesworth, Baber et al. 1992). As the remaining adherent cells proliferate, the extensive proliferative capacity of the initially small stem cell population results in an increasing predominance of stem cells in the final preparation. Another technique includes the use of Percoll or Ficoll, which are high density solutions with low viscosity and low osmotic pressure, to separate distinct cell sub-populations from aspirates. After collection, the BM aspirate is placed on a density solution. Following centrifugation, the cells at the interface between the plasma and the gradient density solution are collected, counted and plated in tissue culture dishes. The interface corresponds to the mononuclear cell (MNC) fraction containing the MSCs (Fortier 2005; Pountos, Corscadden et al. 2007). Bourzac and colleagues showed that the initial

yield of nucleated cells using the Classic protocol was higher than with Ficoll and Percoll isolation protocols. In the same study, the Percoll method resulted in a six-fold increase in BM-MSCs after 14 days of monolayer culture, in comparison to the numbers obtained with the Classic protocol. However, cell counting at P1 showed no significant differences in the yield of BM-MSCs between the three isolation protocols used (Bourzac, Smith et al. 2010). Additionally, passage 1 BM-MSCs isolated by the Classic, Ficoll or Percoll methods, showed similar osteogenic, chondrogenic and adipogenic differentiation capacities (Chang, Hsieh et al. 2009; Bourzac, Smith et al. 2010). Another isolation protocol uses an ammonium chloride-based red blood cell (RBC) lysis buffer to clear these cells from the aspirate and retain the nucleated cell population. After collection of BM sample, the aspirate is mixed with ammonium chloride, incubated at room temperature and the erythrocytes are removed by lysis. The RBC lysis buffer protocol results in larger colony formation than the Classic and Ficoll protocols. In the Classic protocol, the thick layer of erythrocytes present during the first 48 hours of culture interferes with colony formation. However, the osteogenic and adipogenic differentiation potential of BM-MSCs at P2 was not affected by Ficoll, Classic or RBC lysis buffer isolation protocols (Horn, Bork et al. 2008; Horn, Bork et al. 2011). These studies indicate that the initial yield of cells from aspirates varies with the isolation protocol, but after one or two *in vitro* passages, neither the cell numbers nor the differentiation potential is affected. Therefore, any of the mentioned protocols can be used for BM-MSC isolation.(Horn, Bork et al. 2008; Horn, Bork et al. 2011).

#### *In vitro* characteristics of MSCs

Under appropriate culture conditions (initial low seeding densities), it is possible to recognize the formation of colonies from proliferation of a single precursor cell, termed a ‘colony-forming-unit’ (CFU) (Friedenstein, Deriglasova et al. 1974; Castro-Malaspina, Gay et al.

1980; Kuznetsov, Krebsbach et al. 1997; Bianco, Riminucci et al. 2001). These colonies are extremely heterogeneous in terms of size, morphology and differentiation potential. The number of CFUs decreases as the cells are expanded in culture, becoming more morphologically homogenous (Digirolamo, Stokes et al. 1999; Ho, Wagner et al. 2008). Based on morphology, spindle-shaped cells present within the colonies indicate a greater potential for expansion. On the other hand, as the cells are passaged, large flat cells with slow replicative ability appears in the monolayer cultures (Mets and Verdonk 1981; Mets and Verdonk 1981; Colter 2000). Other studies have distinguished the presence of a third small round-cell subpopulation called ‘rapidly self-renewing cells’ (RS cells). It has been proposed that RS cells represent particularly multipotent progenitors with the potential to differentiate into many lineages. However, RS cells were reported only when cells were plated at very low densities (three cells/cm<sup>2</sup>). Additionally, their identification by various surface epitopes is not possible (Colter, Sekiya et al. 2001; Prockop, Sekiya et al. 2001). It seems likely that the RS cells described in these studies may not represent a pure progenitor fraction but represent a variation in the morphology of MSCs.

The presence of CFUs *in vitro* reflects the ability of MSCs to replicate; although this proliferative capacity is limited; MSCs are not immortal (McCulloch, Strugurescu et al. 1991; Bruder, Jaiswal et al. 1997). Following a variable number of cell divisions, MSC enter a state of senescence which is characterized not only by morphological changes (large flat cells), but also a reduced, even absent, proliferative capacity (Digirolamo, Stokes et al. 1999; Wagner, Horn et al. 2008). It has been reported that human MSCs maintain their capacity to differentiate into osteoblasts after 15 passages in culture. However, fewer colonies develop over time, and these cells lose their ability to differentiate (Bruder, Jaiswal et al. 1997). In agreement with the previous study, Digirolamo et al showed that, after 12 passages in culture, the ability of human

MSCs to differentiate into adipocyte and osteoblast lineages, was reduced (Digirolamo, Stokes et al. 1999). It is also important to note that the *in vitro* replicative potential of MSCs varies between donors, and is particularly influenced by donor age. Put simply, the older the donor age, the lower the number of osteoprogenitor cells present in the BM (Majors, Boehm et al. 1997). Fewer CFUs positive for ALP expression are present in BM samples from older animals and humans (Quarto, Thomas et al. 1995; Bergman, Gazit et al. 1996; D'Ippolito, Schiller et al. 1999; Muschler, Nitto et al. 2001).

It has been postulated that the addition of basic fibroblastic growth factor (bFGF) to the culture medium during monolayer expansion can augment the proliferation of MSCs and retain subsequent differentiation potential (Mastrogiacomo, Cancedda et al. 2001; Tsutsumi, Shimazu et al. 2001; Bianchi, Banfi et al. 2003; Stewart, Byron et al. 2007). Martin and co-workers reported that the *in vitro* expansion of primary human BM-MSCs with bFGF increased the growth rate and size of CFUs while maintaining a spindle-shaped phenotype. In the same study, BM-MSCs at P1 treated with osteogenic media but without bFGF, increased their osteogenic potential compared with the untreated BM sample. On the other hand, when BM-MSCs in osteogenic media were constantly exposed to bFGF, their osteogenic ability decreased (Martin, Muraglia et al. 1997). These results suggest that some MSCs remain in an undifferentiated state while others differentiate into the induced pathway. Other studies have demonstrated that bFGF enhances the chondrogenic differentiation of MSCs and improves the ability of MSCs to repair cartilage defects *in vivo* (Hiraki, Shukunami et al. 2001; Henson, Bowe et al. 2005). These studies showed that local administration of bFGF to a large cartilage defect stimulated a selective group of chondrogenic MSCs within the joint, filling the defect with hyaline cartilage. Another study conducted by Solchaga et al, demonstrated that human BM-MSCs expanded in monolayer

with bFGF showed a faster proliferation rate, compared with control groups (Solchaga, Penick et al. 2005). As a result of these studies, bFGF has become widely used in MSC monolayer cultures to shorten the period of time in culture and to increase the cell yield without affecting the cells' differentiation potential. Generating a high number of MSCs in a short period of time is critical for future clinical application in MSC-based therapies (Tsutsumi, Shimazu et al. 2001; Solchaga, Penick et al. 2005).

The capacity of MSCs to differentiate into multiple lineages of differentiated cells is considered a standard assay for *in vitro* MSCs characterization (Dominici, Le Blanc et al. 2006). The most common assays used to assess the differentiation capacity of cultured MSCs are the osteogenic, adipogenic and chondrogenic lineages. For osteogenic induction, standard growth media containing D-MEM, fetal bovine serum and antibiotics, is supplemented with dexamethasone,  $\beta$ -Glycerophosphate and ascorbic acid (Jaiswal, Haynesworth et al. 1997; Pittenger, Mackay et al. 1999; Ogura, Kawada et al. 2004). Following 2-3 weeks of monolayer culture, differentiation is monitored by the presence of cell aggregates that form dense nodules, seen by phase-contrast microscopy (Bellows, Aubin et al. 1986). The assays used to characterize the osteogenic phenotype includes Alizarin Red and Von Kossa staining to detect the presence of mineralized matrix, alkaline phosphatase (ALP) activity, and increased expression of osteoblast-specific transcription factors such as runx2 (or Cbfa1) and osterix, and osteoblast-specific proteins such as osteonectin, osteopontin, osteomodulin, bone sialic protein and ALP (Jaiswal, Haynesworth et al. 1997; Ogura, Kawada et al. 2004).

The International Society of Cellular Therapy (ISCT) proposed that human MSCs exhibit the following properties: plastic adherence, proliferative capacity, multi-lineage differentiation and expression of specific cell surface markers. The ISCT stated that “human MSCs should

express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLA class II surface molecules” (Dominici, Le Blanc et al. 2006). In equine BM-MSCs, it is possible to characterize CD90, CD44, CD29 stem cell surface markers (Arnhold, Goletz et al. 2007; Radcliffe, Flaminio et al. 2010). Ibrahim and co-workers tested three hundred and seventy-nine monoclonal human antibodies against equine leukocyte surface antigens. By flow cytometric analysis, it was possible to identify only 14 human antibodies that cross-reacted with equine epitopes (Ibrahim, Saunders et al. 2007). Many attempts have been made to characterize and identify MSCs by cell surface markers, with some success. However, the limited availability of species-specific monoclonal antibodies as well as the lack of reactivity of human and other species reagents to equine epitopes limit the use of immune-phenotyping in horses (Taylor, Smith et al. 2007).

## **OSTEOGENESIS**

### Bone formation during embryonic development

The first event to occur during skeletogenesis is the migration and condensation of undifferentiated cells into areas of future skeletal elements (Young 1962). This migration process generates a cartilaginous anlagen or template for the formation of the future axial and appendicular skeletal elements (Hall and Miyake 1992). Following condensation, the mesenchymal cells begin to differentiate along the chondrocytic pathway. The resultant chondrocytes secrete aggrecan, collagen type II and other molecules, forming an extracellular matrix characterized as hyaline cartilage (Cancedda, Castagnola et al. 2000). As development continues, pre-determined chondrocytes terminally differentiate into hypertrophic chondrocytes



in the central diaphysis of long bones (primary ossification center), epiphyses and apophyses (secondary ossification centers). Following hypertrophy, chondrocytes undergo apoptosis coincident with vascular invasion from the perichondrium. Through the blood vessels, the perichondrium that surrounds the future diaphysis is transformed into periosteum and osteoprogenitor cells migrate into the vascularized cartilage matrix, mature into osteoblasts and begin to deposit bone matrix (Ducy 2000). This process involving stem cell condensation, chondrogenesis, cartilage hypertrophy, degradation, and replacement by bone is collectively called 'endochondral ossification' (Erlebacher, Filvaroff et al. 1995; Olsen, Reginato et al. 2000; Seibel 2006). Secondary ossification centers, the epiphyses, are formed at each end of growing bones. As the diaphysis and epiphyses ossify, chondrocytes between the epiphyseal ossification centers and the bone shaft are organized into a structure termed the growth plate cartilage. The growth plate is responsible for longitudinal skeletal growth of the bone until sexual maturity (Brighton 1978; Hunziker 1988; Farnum and Wilsman 1993; Hunziker 1994). As the newly formed bone matrix mineralizes, osteoclast precursors from the blood stream and from hematopoietic cells located in the bone marrow (Udagawa, Takahashi et al. 1990), invade the center of the ossifying structure and begin to differentiate. The mature osteoclasts resorb mineralized matrix to form the internal space that will constitute the bone marrow cavity. Osteoclasts are phagocytic cells derived from monocytes that are uniquely capable of resorbing mineralized matrix and are required for bone resorption and subsequent remodeling (Suda, Takahashi et al. 1992; Lanyon 1993; Athanasou 1996).

Another mechanism of bone formation involves 'intramembranous ossification', whereby mesenchymal cells condense, the tissue vascularizes and cells differentiate directly into osteoblasts without the intermediate generation of a cartilaginous template (Seibel 2006). This

type of ossification is characteristic of most of the surface bones of the skull and is also involved in the deposition of new bone to the outer periosteal surfaces of long bones.

### Bone Extracellular Matrix

Osteoprogenitor cells, located in the BM together with hematopoietic stem cells, give rise to and maintain populations of pre-osteoblasts and osteoblasts, osteocytes within the bone matrix, and the so-called lining cells located in the inactive surface of bone (Friedenstein 1976; Heng, Cao et al. 2004). Osteoblastic cells are responsible for producing the components of the bone matrix including type I collagen (85% of matrix dry weight) and type V collagen (5%) (Niyibizi and Eyre 1989; Hadjidakis and Androulakis 2006). In addition, there are other non-collagenous components (comprising 10% of the matrix dry weight) such as osteopontin (OSP), bone sialoprotein (BSP), osteocalcin (OC), osteonectin (OSN) and osteomodulin (OSM). The inorganic portion of bone is composed by crystals of hydroxyapatite, interposed within the collagen fibers of the bone matrix (Herring and Ashton 1974).

Osteonectin (OSN), also termed SPARC, was initially isolated from bone but later was also localized in many different types of tissues undergoing remodeling processes. In bone, OSN is synthesized by osteoblasts and is one of the most abundant non-collagenous extracellular matrix proteins (Termine, Kleinman et al. 1981; Wasi, Otsuka et al. 1984; Young, Bolander et al. 1986; Holland, Harper et al. 1987; Wewer, Albrechtsen et al. 1988; Brekken and Sage 2001). Choi and co-workers cultured the neonatal mouse osteoblastic MC3T3-E1 cell line under osteogenic conditions for 28 days. At the transcriptional level, OSN mRNA was expressed at day 10 and the level of expression was maintained throughout the culture period, peaking between days 10 and 16, concurrent with the nodule formation at day 16. Following matrix

mineralization, OSN expression was down-regulated (Choi, Lee et al. 1996). The same results were obtained in another study using mouse embryonic stem cells under the same culture conditions and period of time in culture (zur Nieden, Kempka et al. 2003). These studies provide evidence that OSN is expressed late in the osteoblast differentiation process, but before mineralization occurs.

Osteocalcin (OC), also called Gla protein, is only present in areas where osteoblastic cells are in close proximity with bone during skeletogenesis. This indicates that OC is synthesized by mature osteoblasts (Boivin, Morel et al. 1990; Weinreb, Shinar et al. 1990; zur Nieden, Kempka et al. 2003). OC null-mice are normal at birth; however, over time, the mutant mice showed increased mineralized matrix content in their long bones. OC acts as a negative regulator of osteogenesis, limiting the formation of bone (Ducy, Desbois et al. 1996). Also, OC functions to recruit mature osteoclasts and osteoclast precursors to bone for matrix resorption (Davies 1996). Since OC is synthesized by osteoblasts and is released from bone matrix by osteoclastic resorption into the circulation, it is considered a biomarker of bone turnover.

Bone sialoprotein (BSP) belongs to the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family, whose members are involved in the development, turnover and mineralization of bone and dentin (Fisher and Fedarko 2003). Chen and colleagues demonstrated that BSP is expressed in mineralized connective tissues early during bone, cementum and dentin formation (Chen, Shapiro et al. 1991; Chen, Shapiro et al. 1992). However, Bianco showed that BSP was also expressed in mature osteoblasts and young osteocytes, by immunohistochemical localization (Bianco, Fisher et al. 1991). In fact, BSP expression is similar to that of osteopontin (Mark, Prince et al. 1987) which is also considered a member of SIBLING family.

Osteopontin (OSP) is a sialoprotein that has the ability to control the mineralization process in bone as well as other tissues such as kidney and epithelial surfaces. In bone, OSP has the ability to link osteoblasts and osteoclasts to crystals of hydroxyapatite in the bone matrix (Oldberg, Franzen et al. 1986; Helder, Bronckers et al. 1993; Giachelli and Steitz 2000). OSP is expressed late during osteoblast differentiation and at the beginning of the mineralization process (zur Nieden, Kempka et al. 2003). OPN-null neonatal mice do not show abnormalities in their skeleton size and patterning, as demonstrated by both light and electron microscopy (Rittling, Matsumoto et al. 1998). However, by 4-6 months of age, OPN-null mice show suppression of osteoclast activity and consequent increased deposition of bone (osteopetrosis) compared with the wild-type mice (Yoshitake, Rittling et al. 1999).

Another proteoglycan present in bone ECM is osteomodulin (OSM), also called osteoadherin. OSM is specifically expressed by mature osteoblasts as demonstrated by *in situ hybridization* studies (Sommarin, Wendel et al. 1998), similarly to BSP (Hultenby, Reinholt et al. 1991). OSM has the ability to link cells to bone ECM (Wendel, Sommarin et al. 1998). It has also been suggested that OSM may act as a signaling factor for maturation during bone matrix deposition (Ramstad, Franzen et al. 2003). To date, its specific function remains unknown.

### Transcriptional regulation of bone formation

Transcription factors are proteins that bind to specific DNA sequences and regulate expression of genes in positive or negative (repressive) fashion (Latchman 1997). During different stages of bone development, multiple transcription factors control the differentiation of osteoblasts. The primary factors that regulate osteogenesis are Osterix (Osx) and Core-binding factor 1 (Cbfa1) -also called Runt related transcription factor 2 (Runx2).

Osterix (Osx) is a zinc finger-containing transcription factor capable of stimulating osteoblast differentiation. Osx-null mice do not develop bone or bone trabeculae through intramembranous or endochondral ossification processes, as evidenced by Alizarin red staining. However, MSCs in these mice are able to migrate, along with osteoclasts and blood vessels, to the sites of future bone formation. In Osx-null mice, the areas that are supposed to ossify by endochondral ossification persist as cartilage, as demonstrated by Alcian blue staining. These mice die immediately following birth, due to respiratory failure. Osx controls the osteoblastic expression of proteins required for bone ECM formation, including osteonectin, osteocalcin, osteopontin, bone sialoprotein and collagen type I (Nakashima, Zhou et al. 2002), emphasizing the importance of Osx in matrix formation and mineralization.

The location and activation of Osx during osteoblast differentiation was traced and visualized in cultured embryonic stem cells (ESC), using an Osx response element cDNA construct fused to green fluorescent protein (GFP). Following 7 days in osteogenic culture and coincident with the formation of MSCs, the Osx-GFP fusion protein was expressed and translocated to the nucleus (Tai, Christodoulou et al. 2005). In addition to stimulating osteogenesis, Osx also prevents the commitment of mesenchymal progenitor cells to other lineages such as chondrogenic or adipogenic pathways. BM-MSCs transfected to overexpress Osx were unable to express PPAR- $\gamma$ , which is an adipocyte-specific transcription factor but did form mineralized nodules *in vitro* without osteogenic factors added into the culture media (Choi, Pratap et al. 2001; Tu, Valverde et al. 2006). These studies indicate the critical function of Osx in the early commitment of MSCs to the osteoblastic lineage.

The core-binding factor (CBF) transcription factors are a family of heterodimeric proteins comprised of two subunits, a DNA-binding  $\alpha$  subunit and a non DNA-binding  $\beta$  subunit. The

CBF  $\alpha$  subunit includes three distinct genes (Cbfa1, Cbfa2 and Cbfa3) that participate in the regulation of genes required for cellular differentiation and development (Banerjee, Javed et al. 2001). Cbfa1 is also called Runx2 because it includes a conserved amino acid domain ( *runt* domain) present in *Drosophila* (Kania, Bonner et al. 1990; Komori 2005). Cbfa1/Runx2 homozygous-deficient mice develop skeletal elements only composed of cartilage that stain positively with Alcian blue. These studies emphasize the importance of Cbfa1/Runx2 for early mesenchymal stem cell migration and early osteoblast maturation for skeletal ossification through both endochondral and intramembranous mechanisms (Komori, Yagi et al. 1997; Nakashima, Zhou et al. 2002). In addition, Cbfa1/Runx2 expression was found to be low in chondrocytes of developing cartilage, almost absent in permanent cartilage but highly expressed in hypertrophic chondrocytes of the growth plate (Inada, Yasui et al. 1999; Kim, Otto et al. 1999). Overexpression of Cbfa1/Runx2 in growth plate chondrocytes stimulates their maturation and endochondral ossification (Ueta, Iwamoto et al. 2001). On the other hand, Cbfa1/Runx2 heterozygous mice demonstrated specific defects resembling cleidocranial dysplasia (CCD) which is a human skeletal disorder that affects the closure of the cranial fontanelles and clavicle formation. In fact, human patients with CCD showed mutations present in the Runx2 gene, confirming that a heterozygous Runx2 mutation is responsible for this disorder (Mundlos, Otto et al. 1997; Otto, Thornell et al. 1997; Ducky, Starbuck et al. 1999)

In Cbfa1/Runx2 null-mice, *Osx* is not expressed during development. However, in *Osx* null-mice, Cbfa1/Runx2 is expressed at levels comparable to wild type mice. These observations confirm that *Osx* acts downstream of Cbfa1/Runx2, inducing pre-osteoblast differentiation into mature osteoblasts, consequent to Cbfa1/Runx2 trans-activation (Nakashima, Zhou et al. 2002).

### Assays for *in vitro* osteogenic differentiation

*In vitro* osteogenic assays are routinely used to assess osteogenic differentiation of different MSC populations. Following a period of time in culture under the appropriate conditions, it is possible to assess alkaline phosphatase activity (ALP) expression and activity and stain for matrix mineralization. ALP is a membrane-bound metalloenzyme also present in a soluble form in body fluids, including blood (Moss 1997). Four isoenzymes exist: intestinal, placental, germ cells and tissue-nonspecific. The tissue-nonspecific ALP (TNAP) has three isoforms, expressed in bone, liver and kidney (Goldstein, Rogers et al. 1980). ALP is commonly used as a biochemical marker to assess osteoblast activity, using p-nitrophenyl phosphatase (p-NPP) as substrate. The hydrolysis of phosphate esters is catalyzed by ALP under alkaline pH conditions. A colored yellow end product, p-nitrophenol, is optically measured at 405 nm wavelength (Bessey, Lowry et al. 1946; Sabokbar, Millett et al. 1994; Akcakaya, Aroymak et al. 2007). In contrast, the mineralization process is considered “a functional *in vitro* endpoint reflecting advanced cell differentiation” (Hoemann, El-Gabalawy et al. 2009). Alizarin red staining is used to detect calcium deposition and Von Kossa staining indicates phosphate deposition within the mineralized matrix (Henrichsen 1956; Puchtler, Meloan et al. 1969).

Other methods to determine osteogenic differentiation of MSCs use transcriptional analyses of specific osteoblastic genes including Cbfa1/Runx2, Osx, Col I, OSN, OC, OPN, OSM, BSP and ALP (Jaiswal, Haynesworth et al. 1997; Carpenter, Goodrich et al. 2010). Generally speaking, ALP expression increases during early osteogenesis, but when mineralization is advanced, its expression decreases; OPN precedes the expression of OSN and BSP, peaking twice during proliferation; BSP is expressed transiently very early and then

upregulated when osteoblasts are fully differentiated; finally OC is expressed concurrently with mineralization of the ECM (Madras, Gibbs et al. 2002).

## **CLINICAL APPLICATION OF MSCs FOR BONE REPAIR**

### **Bone remodeling and repair**

In vertebrates, the bone formation process starts during fetal development and continues throughout life as remodeling and repair processes (Athanasou 1996; Hadjidakis and Androulakis 2006). During the process of remodeling, osteoclasts remove mineralized bone matrix and this is followed by osteoblastic new bone matrix deposition (Frost 1969). In this way, the shape, quality and size of bone is maintained and constantly adjusted to the stresses and biomechanical forces experienced by the skeleton (Hadjidakis and Androulakis 2006). In addition, the bone remodeling process is active in repair following an injury such as fracture (Ferguson, Miclau et al. 1998). This process is similar to the bone formation occurring during skeletal development (Bruder, Fink et al. 1994; Kraus and Kirker-Head 2006). In contrast to soft tissue injuries, where the healing process involves the formation of a scar tissue, bones actually have the ability to heal by new bone formation, an authentically regenerative process (Ferguson, Alpern et al. 1999).

Most fractures are repaired by secondary bone healing, since some level of mechanical instability exists at the fracture site. Four stages of fracture repair have been described (Schindeler, McDonald et al. 2008).



- 1) The first response when a fracture occurs is inflammation associated with soft tissue and vascular damage.
- 2) Chondrocytes and fibroblasts within the fracture site provide temporary mechanical stabilization through the formation of a soft callus
- 3) A hard callus is formed by active osteoblasts that mineralize the callus matrix
- 4) The irregular hard callus is remodeled by osteoclastic resorption and osteoblastic deposition of new bone.

In this way, the fracture fragments are fused, and the bone recovers its original shape over a variable period of time (between six to twelve weeks), depending on the severity of the fracture and displacement of the bone fragments (Ham 1952).

Primary bone healing is only possible when the fracture site is stabilized and compressed, whether by screws or compression plates. Callus formation is not necessary and direct bone formation and remodeling occurs over a shorter period of time (Ashhurst 1986).

#### Clinical application of MSCs

The physiologic bone repair process after a fracture occurs is usually sufficient when appropriate mechanical support exists. On the other hand, under certain circumstances, it becomes necessary to augment or enhance the natural healing process of bone repair (Kraus and Kirker-Head 2006). The circumstances that may require additional intervention are: significant loss of bone as a result from trauma or a tumor resection, and, in horses particularly, to enhance arthrodesis techniques and treat osseous cyst-like lesions.

In horses, the common reasons of failure in fracture repair are infection, delayed union, and implant failure. The more important consequence of delayed union is the high incidence of lameness and overload of the contralateral limb, resulting in laminitis in adults and hyperextension deformities in young horses (Virgin, Goodrich et al. 2011). As a consequence, the prognosis for return to function is lower than in other species.

Many cell-based therapies are available to enhance bone repair in humans as well as in large animal orthopaedics. Autologous bone grafts or bone autografts (BA) have been widely used to aid in the fracture repair. However, the collection of an autograft has limitations for its clinical use. The site where the BA is harvested requires a separate surgical access, therefore causing donor site morbidity, extending surgical time and increasing the risks for wound infection (Damien and Parsons 1991). Also, the amount of BA that can be collected may be insufficient for applications in large bone defects. Moreover, in aged animals, the population of osteoprogenitor cells in autograft may be reduced, leading to an unsuccessful transplant activity (Frodel, Marentette et al. 1993; Boone 2003).

Since the development of MSC biology and regenerative medicine, many researchers have worked on developing new techniques for stem cell applications in bone regeneration. To mention some examples, Ohgushi and colleagues showed in a rat model that the combination of BM-MSCs immersed in a porous calcium phosphate ceramic implant served to heal a critical diaphyseal bone defect (Ohgushi, Goldberg et al. 1989). Similar results, using implants loaded with BM-MSCs, were obtained in critical-sized defects in the long bones of adult dogs (Bruder, Kraus et al. 1998) and sheep (Marcacci, Kon et al. 1999; Kon, Muraglia et al. 2000). In humans, bone nonunions have been successfully treated with the percutaneous injection of autologous

BM-MSCs (Healey, Zimmerman et al. 1990; Connolly, Guse et al. 1991). Clearly, these strategies are adaptable to use in equine orthopaedic conditions.

The experiments in this study were carried out to compare the osteogenic capacities of putative mesenchymal stem cell populations from equine synovium, bone marrow and adipose tissue. The study was designed to address the following questions:

1. Does cell 'source' influence the expression of osteogenic markers under basal culture conditions? Specifically, do BM-MSCs express higher levels of osteogenic markers and activities that 'pre-commit' or prime these cells for osteogenesis?
2. Are these nominal stem cell populations, isolated and expanded using standard protocols, capable of osteogenic differentiation by both qualitative and more rigorous quantitative criteria?
3. What are the comparative osteogenic activities of these cell populations?

The overall hypothesis tested by the study was as follows:

SYN-MSCs, BM-MSCs and AD-MSCs have the same capacity for *in vitro* osteogenic differentiation, under identical culture conditions.

## CHAPTER 3

### MATERIALS AND METHODS

#### Collection of samples

Bone marrow, adipose tissue and synovium were collected aseptically from six healthy two-year-old horses. Horses were sedated with an alpha-2 agonist (0.5 to 1.0 mg of xylazine/kg administered IV). An IV catheter was placed in the jugular vein and anesthesia was induced with 2.2 mg of ketamine/kg and 0.1 mg of diazepam/kg. For maintenance, a combination of 5% guaifenisin solution with 1,000 mg of ketamine/L and 1,000 mg of xylazine/L was used. Following collection of bone marrow aspirates, all horses were euthanized with 104 mg of sodium pentobarbital/ kg given IV. The adipose tissue and synovium were collected immediately following euthanasia.

For bone marrow collection, a 5cm x5 cm area over the tuber coxae was clipped and aseptically prepared. A stab incision was made with a #11 scalpel blade at the site of collection. The bone marrow aspirate was obtained through a bone marrow biopsy needle. The 10-15 ml of aspirate was collected into 30 ml syringes containing 1,000 IU of heparin to prevent coagulation, and this was diluted to a volume of 50 ml with PBS solution.

Adipose tissue was collected from the subcutaneous AD depot lateral to the tail head. A 10-15 cm skin incision was made and 8-10 grams of AD were collected and placed in a 50 ml polypropylene tube containing sterile PBS solution.

For synovium collection, the skin over the right radiocarpal joint was clipped and aseptically prepared. An incision was made into the dorsal aspect of the joint and the subsynovial

inner capsule of the joint was exposed. About 2-3 grams of synovium was harvested and placed into a 50 ml polypropylene tube containing sterile PBS solution.

### Cell isolation

The isolation of bone marrow-derived stem cells was achieved by first centrifuging 10-15 ml of the aspirated bone marrow sample, diluted with 5-10 ml of PBS solution, at 300g for 15 minutes. The cell pellet was washed with PBS and re-centrifuged as described. The supernatant was removed and the cell pellet was re-suspended with 0.8% ammonium chloride to lyse the red blood cells.

The adipose tissue was diced into small pieces and digested for 3 hours at 37 ° C with 0.2 % collagenase (type II; Worthington Biochemical Corporation) in 10 ml of Alpha-MEM/gram of tissue and 2% penicillin/streptomycin. After digestion, cells were filtered through a 40 µm mesh filter.

The isolation of synovium-derived stem cells was performed by a technique previously described (He, Chen et al. 2009). Briefly, synovial tissue was diced and digested at 37 ° C for 30 minutes with 0.1% trypsin. Subsequently, 0.1% collagenase in 10 ml of DMEM/gram of tissue was used for 2 hours to release the cells.

The cells isolated from each source were collected by centrifugation. The cell numbers of primary adipose and synovial cells were determined by using a hemacytometer. Additionally, cellular viability was assessed by trypan blue exclusion followed by cryo-preservation until further use for monolayer expansion and osteogenic induction. Primary bone marrow-derived cells were plated and cultured until the primary monolayers reached 80% confluence. The cells were then trypsinized, seeded at  $5 \times 10^3$  cells per  $\text{cm}^2$  and expanded for one passage before cryo-

preservation. For all three cell types, the medium used for cryopreservation contained 50 % DMEM, 40 % FBS and 10 % Dimethyl sulfoxide (DMSO). The cryovials were placed in an isopropanol freezing chamber at -80 ° C for 24 hours before being transferred to liquid nitrogen.

### Monolayer expansion

The basal growth medium used for monolayer expansion was Dulbecco's modified Eagle's medium [DMEM], supplemented with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin. Additionally, basic fibroblastic-growth factor (bFGF) was used to stimulate cell proliferation and shorten the period of time required for monolayer expansion over two passages. The cells were seeded at a density of  $5 \times 10^3$  cells per  $\text{cm}^2$  in 100 mm plates ( $55 \text{ cm}^2$ ). After 48 hours, the culture media was changed to remove non adherent-cells. Thereafter, the media was changed three times per week. The cells were incubated at 37 °C, 5 %  $\text{CO}_2$  and 90 % humidity. When cells reached 80% confluence, the monolayers were rinsed with PBS solution and 2 mL of 0.05% Trypsin-EDTA was added to release the cells. Following 3-4 minutes incubation at 37 °C, 10 mL DMEM was added to deactivate the Trypsin-EDTA. The cell suspension was transferred to 15 mL tube and centrifuged for 8 minutes at 900 rpm. The supernatant was discarded and the cells were re-suspended in 10 mL DMEM. The cell numbers were calculated by counting an aliquot of the resulting suspension using a hemacytometer and inverted light microscope. Briefly, a 1:10 dilution of the cell suspension and 10% Trypan blue dye were prepared in two microcentrifuge tubes. The samples were mixed and 10  $\mu\text{L}$  from each tube were added to each side of the hemacytometer. The total number of cells and the viability were calculated by the following formulas:

**# of cells** = # cells counted / # squares x  $10^4$  (Hemocytometer) x 10 (cell dilution) x 10 (total volume of cell suspension)

**Viability** = # total live cells / # dead cells + # total live cells

#### Induction of osteogenic differentiation

Osteogenic differentiation of each cell population was induced by culturing passage 3 cells in osteogenic medium for 14 days. The basal growth medium consisting in DMEM, 10% FBS and antibiotics was supplemented with 100 nM Dexamethasone (Sigma-Aldrich), 1mM  $\beta$ -Glycerophosphate (Sigma-Aldrich) and 50  $\mu$ g/ml ascorbic acid (Wako, Japan). After 7 and 14 days in culture, control and osteogenic cells were harvested for RNA isolation and Alkaline Phosphatase (ALP) bio-activity. Additionally, colorimetric assays were performed to detect mineral deposition and ALP activity within the cell monolayers (**Figure 1**).

#### RNA isolation

Total RNA was isolated from the cell monolayers using the phenol-based dissociation agent, TRIzol<sup>®</sup> (Invitrogen Corporation, Carlsbad, CA). One milliliter of TRIzol<sup>®</sup> was added into each well. The cell lysates were scraped from the cell surface, transferred to 15 ml falcon tubes and stored at -80°C. An Ultra Turrax-T 25 homogenizer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) was used to disrupt the cells. Following a 3-minute centrifugation, the cell lysates were transferred to 1.5 ml microcentrifuge tubes and 200  $\mu$ L chloroform added. Samples were vortexed and incubated for 2-3 minutes at room temperature. Following centrifugation at 12,000 rpm for 30 minutes at 4°C (Centrifuge 5415 R, Ependorf), the upper aqueous phase (approximately 400  $\mu$ L) containing the RNA was transferred to a new 1.5 ml microcentrifuge

tube. During this process, great care was taken to leave the insoluble material at the interface undisturbed. This interface material contains insoluble proteins, lipids and DNA.

The RNA within the aqueous phase was precipitated by adding 250 µL of isopropanol and 250 µL of 0.8 M sodium citrate/1.2 M sodium chloride; the ‘high salt’ variation of the basic TRIzol protocol, used for samples containing high levels of proteoglycans. The samples were stored overnight at -20 °C to facilitate RNA precipitation. The microcentrifuge tubes were then centrifuged at 10,000 rpm for 20 minutes at 4°C, resulting in the formation of a translucent pellet. The supernatant was removed and the pellet was washed in 500 µL of 70% ethanol (to reduce the salt content of the pellet). The samples were vortexed, incubated for 10 minutes at room temperature and centrifuged 5 minutes at 10,000 rpm. The supernatant was removed and the pellet was allowed to air-dry for 5-10 minutes. A small volume of diethyl pyrocarbonate (DEPC)-treated water was added to dissolve the pellet. The samples were kept on ice for 5 minutes, vortexed, spun down to bring contents to the bottom and incubated at 65 °C for 10 minutes. The concentration of RNA (1:40 aliquot of each sample) was determined by measuring the absorbance at 260 nM (A260) and 320nM (A320) in a spectrophotometer (Smart Spec™ 3000, BioRad, Hercules, CA). Total RNA was calculated as follows:

$$\text{RNA in } \mu\text{g}/\mu\text{L} = \frac{\text{OD at 260 nM} - \text{OD at 320 nM} \times 40 \text{ (dilution factor)} \times 40 \text{ (coefficient)}}{1000}$$

### Reverse transcription

A commercially available reverse transcription kit (Superscript™ First-Strand Synthesis System for RT-PCR, Invitrogen) was used to generate cDNA. Briefly, the volume of each sample containing 1 microgram of total RNA was calculated and brought up to 8 µL total with



DEPC water. A master mix (2  $\mu$ L per sample) containing 1  $\mu$ L oligo dT and 1  $\mu$ L 10 mM dNTPs was added into each tube. Following incubation in a 65 °C water bath for 5 minutes, the samples were placed on ice for 1 minute to facilitate binding of the oligonucleotides. A master mix (9  $\mu$ L per sample) containing 2  $\mu$ L 10x First Strand Buffer, 4  $\mu$ L 25 mM MgCl<sub>2</sub>, 2  $\mu$ L 0.1 M DTT and 1  $\mu$ L RNase OUT was added into each tube. Samples were incubated in a 42 °C water bath for 2 minutes and 1  $\mu$ L (50 units) SuperScript II Reverse Transcriptase (RT) was added. The samples were then incubated at 42 °C water bath for 50 minutes. The reaction was stopped by transferring the sample to a 70° C water bath for 15 minutes. 1  $\mu$ L of *RNase H* was added to remove the RNA from the sample, leaving only cDNA. Following the incubation at 37 °C for 20 minutes, the samples were placed on ice and 181  $\mu$ L of distilled water (dilution 1:10) was added, vortexed and stored at – 20 °C until further use.

### PCR amplification

The relative expression of osteogenic genes including alkaline phosphatase (Alp), osterix (Osx), osteonectin (Osn), Osteomodulin (Osm), runt-related transcription factor 2 (Runx-2) and the reference gene, elongation factor -1 alpha (EF1- $\alpha$ ) was assessed by quantitative real-time PCR (qPCR) in both control and osteogenic samples. The primers used for qPCR analysis are listed in **Table 1**.

Lyophilized primers were reconstituted in 1X TE buffer to generate 100  $\mu$ M stock solution which was then diluted 1:10 to yield 10  $\mu$ M working stocks. The total volume used for the PCR reaction was 25  $\mu$ L; therefore, the final concentration of each primer used was 0.4  $\mu$ M (or 400 nM). All primers were designed for a nominal 60° C annealing temperature, but all were tested to determine the optimum conditions for each PCR reaction. Briefly, a cDNA positive

control was amplified (BioRad thermocycler, iCycler iQ<sup>TM</sup>) using a gradient of annealing temperatures (64°C, 62.1°C, 60.3°C, 57.7°C and 54.8°C) with *Taq* DNA polymerase and the specific primers described. Following PCR amplification, 5 µl of each sample was loaded into a 2% agarose gel and electrophoresed to separate the PCR products. For all primers, the optimum annealing temperature was determined to be 57.7°C (**Figure 2**).

Quantitative real time-PCR (qPCR) was performed using 5 µL of diluted cDNA template (1:10 dilution) combined with 20 µL of a mixture composed of 12.5 µL 1 x SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1 µL each of the 10 µM forward and reverse primer stocks and 5.5 µL DNase/RNase-free water in a 96-well microplate. Each sample was run in duplicate. The reactions were performed in a BioRad iCycler iQ<sup>TM</sup> using the following conditions: initial denaturation for 3 minutes at 95 °C, 40 cycles of denaturation at 95 °C for 10 seconds, annealing temperature of 57.7 °C for 30 seconds and polymerase extension at 72 °C for 20 seconds. The samples were denatured at 95 °C for 1 minute before starting the melting curve protocol which consisted of increasing the temperature from a starting point of 55 °C for 1 minute followed by increments of 0.5 °C every 10 seconds until 95 °C was reached. The presence of a single PCR product was monitored by melting curve analyses. Sterile water was used as a 'no template' negative control for each of the PCR reactions to monitor the possibility of contamination. The qPCR data were normalized to expression of the reference gene, elongation factor -1 alpha (EF1- $\alpha$ ) which was selected after comparisons with expression profiles of  $\beta$ -Actin and -glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with particular regard for inter-sample consistency (**Figure 3**). The level of expression for each target gene was calculated as  $2^{-\Delta Ct}$  and the comparative  $\Delta Ct$  method was used to determine relative gene expression levels, using the BM day 7 Control value as the nominal reference level.

### Pico Green Assay

DNA content was measured using the Pico Green DNA kit (Quanti-iT<sup>™</sup> PicoGreen dsDNA, Invitrogen). Samples were diluted 1:5 in 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). Serial dilutions of calf thymus DNA were used to generate a standard curve. Duplicate 100 µl aliquots of each sample and the standard were transferred to a black 96-well microplate. On the day of the experiment, the Pico Green reagent was prepared, based on the 100 µl volume required for each well (1 µl Pico Green reagent diluted in 200 µl of 1X TE buffer) and added to every sample and standard. The microplate was placed in the dark to prevent reagent photo-degradation. Following 5 minutes incubation, the fluorescence was measured at 485 nm wavelength (FLUOstar OPTIMA, BMG, Lab Technologies).

### Alkaline Phosphatase Bio-activity

Alkaline phosphatase activity was assessed in triplicate samples of control and osteogenically-induced cultures. For each treatment group, cells were plated at  $5 \times 10^3$  cells per  $\text{cm}^2$  in 12 well plates. At days 7 and 14 of culture, the cells were harvested in 1 ml of lysis buffer containing 20mM Tris HCl, 150mM NaCl and 1% Triton X-100. Samples were stored at  $-80^\circ\text{C}$  until further processing. Each sample was homogenized using an IKA Labortechnik T 25 basic homogenizer (Janke & Kunkel GmbH and Co., Staufen, Germany), centrifuged at 2500 rpm for 15 minutes at  $4^\circ\text{C}$  and kept on ice for 30 minutes. Subsequently, the supernatants were assayed for alkaline phosphatase activity using an AP assay kit (Wako, Japan), following the manufacturer's instructions. A substrate tablet containing *p*-Nitrophenylphosphate was dissolved into 5 ml of buffer solution provided (working assay solution). A 100 µL aliquot of working assay solution was transferred into each well of a transparent 96-well microplate. Then, 20 µL of

each sample and standard of *p*-Nitrophenol solution was run in duplicate. The substrate *p*-Nitrophenylphosphate is hydrolyzed into *p*-Nitrophenol by alkaline phosphatase (Bessey, Lowry et al. 1946). The reaction generates a yellow product, optically measured at 405 nm wavelength. The enzymatic activity was determined using the following formula:

$$\text{Activity (units / } \mu\text{l)} = \frac{C}{RT} \times \text{DF}$$

One unit of enzyme activity = release of 1 nmol of *p*-Nitrophenol per minute

C: Concentration of *p*-Nitrophenol released by sample relative to the standard curve (mmol/L or nm/ $\mu$ l)

RT: 10 minutes was used as reaction time

DF: dilution factor of sample

The relative activity of each sample was determined by normalizing the results of each triplicate sample to its corresponding DNA content. Then, a mean and standard deviation were calculated for each triplicate sample.

#### Alkaline Phosphatase staining

To localize alkaline phosphatase activity in osteogenic cell monolayers, a histochemical staining kit was used, following the instructions of the manufacturer (Procedure No. 86. AP, leukocyte; Sigma Aldrich). Briefly, cell layers were fixed with citrate-acetone-formaldehyde fixative solution for 1 minute followed by 3-4 time washes with distilled water. An alkaline dye mixture consisting of a diazonium salt solution and naphtol AS-BI alkaline solution was added to the cell layer and incubated in reduced light conditions at 18°C – 26°C for 15 minutes. Following 3-4 washes with distilled water, the cell layers were counterstained with neutral red solution for 5

minutes. Cell clusters exhibiting alkaline phosphatase activity were marked by blue staining. Representative pictures of the monolayers were obtained from the triplicate samples (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

#### Von Kossa staining

To localize phosphate deposition in the cell monolayers, a Von Kossa stain kit (American MasterTech) was used in triplicate samples of control and osteogenically-induced cultures to identify ionized phosphate. The Von Kossa reagent is a metal substitution stain in which silver ions react with phosphate. Under light illumination, silver phosphate is converted to silver.

Following 30 minutes fixation with 10% formalin, the cell layers were washed 2-3 times with distilled water. One ml of 5% silver nitrate solution was added to each well and exposed to a strong light for 30 minutes. The cell layers were washed 2-3 times with distilled water and 1 ml of 5% sodium thiosulfate was added to remove excess silver salts. Following 3-5 minutes, the cell layers were washed 2-3 times with distilled water. Finally, a neutral red solution was added for 5 minutes as a counterstain. The calcium deposits in the ECM were evident as dark brown or black deposits while the cell cytoplasm stained pink and the nuclei stained red. Representative images of stained monolayers were obtained (Leica Microsystems, Leica Application Suite - LAS- version 2.6.R1).

#### Alizarin Red staining

A 2% Alizarin Red solution was used in triplicates cell layers (control and osteogenically-induced cultures) to identify calcium deposition. Alizarin Red forms complexes with calcium ions.

Following fixation with 10% formalin for 30 minutes, cell layers were washed 2-3 times with distilled water. One ml of 2% fresh Alizarin Red (Sigma-Aldrich) solution (pH 4.1) was added to each well. Following incubation at room temperature for 20 minutes, the stain was removed and washed 3-4 times with water until the rinsed solution was clear. Mineral deposits within the cell layers were stained bright red. Representative pictures of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1).

### Statistical analyses

Mean  $\pm$  SE values were calculated for each cell type at day 7 and 14 in culture with and without osteogenic induction media supplementation. The effect of cell type was analyzed by use of general linear model with horse as a random effect. Among the cell types (SYN-, AD- and BM-MSCs), the effect of supplementation with osteogenic medium over time (day 7 and 14) was evaluated by use of a two-way repeated measures ANOVA to control for differences among horses. When group differences for osteogenic induction were detected, pairwise multiple comparisons were made by use of the Holm-Sidak nonparametric test. A commercially available statistical program (Sigma Stat<sup>®</sup> version 11.0) was used to perform statistical analysis. Values of  $P < 0.05$  were considered significant (**Table 2 to 4**)

## CHAPTER 4

### RESULTS

The experiments in this study were carried out to define and compare the osteogenic capacities of equine MSCs derived from synovium, bone marrow, and adipose tissue, maintained under the same culture conditions. The overall hypothesis tested by the study was as follows:

**SYN-MSCs, BM-MSCs and AD-MSCs have the same capacity for *in vitro* osteogenic differentiation, under identical culture conditions.**

The primary measures used to monitor osteogenesis were matrix mineralization (Alizarin Red and Von Kossa staining) and ALP up-regulation (mRNA levels and enzymatic activity). The secondary indicators of osteogenic differentiation were up-regulation of osteoblast-specific genes Runx2, Osterix, Osteonectin and Osteomodulin. Within the context of this hypothesis, the outcome measures were used to answer three specific questions.

1. Was the expression of osteogenic markers and activities by SYN-MSCs, BM-MSCs and AD-MSCs under basal (non-induced) culture conditions equivalent?
2. Within each cell source (synovium, bone marrow and adipose tissue), was there statistically significant evidence of osteogenic differentiation under appropriate culture conditions?
3. What were the comparative osteogenic differentiation capacities of the three cell sources?

#### Morphological characterization of equine MSCs

The adherent cells isolated from bone marrow, synovium and adipose tissue were heterogeneous in each case, and initial cell proliferation generated colonies of different sizes. All

large colonies consisted of small spindle-shaped cells. In contrast, the small colonies contained cells with a large polygonal shape.

During the primary culture expansion, the BM monolayers took several days longer to reach confluence than the SYN and AD cultures. This is very likely a reflection of the fact that the initial seeding densities of proliferation-competent cells within the BM specimens were somewhat lower than the 5,000 cells/cm<sup>2</sup> densities in the SYN and AD cultures, but the comparative CFU assessments were not made in this study. In subsequent passages, the proliferation rates of all three cell types were very similar.

No obvious differences were apparent in the morphologies of SYN, BM or AD cells during monolayer expansion through two passages. However, within 7 days of culture under osteogenic conditions, the appearance of the monolayers changed dramatically, with the development of dense, multicellular aggregates and a transition of the cells immediately surrounding these aggregates into a more cuboidal shape. The number and density of the BM-MSC aggregates were substantially greater than in the SYN-MSC or AD-MSC cultures at both early (day 7) and later (day 14) time-points (**Figure 4-5**).

#### Matrix mineralization (Alizarin red and Von Kossa staining)

After 14 days in P3 monolayer culture, the cells under control conditions did not show cell aggregation or spontaneous mineralization of the extracellular matrix. In contrast, after 7 days in osteogenic medium, all three cell types developed dense cellular aggregations; these were larger, denser and more abundant in BM-MSC cultures. With Alizarin Red application, the cellular aggregates in the BM cultures showed intense label up-take. The background staining was similar to that of control monolayers, suggesting that matrix mineralization was restricted to



the aggregates (**Figure 6-7**). Alizarin Red staining of osteogenic SYN and AD cultures was also limited to the locations of cellular aggregation but was noticeably less intense. The results of Von Kossa staining were consistent with those of the Alizarin Red staining experiments in all three cell types (**Figure 8-9**).

#### Alkaline Phosphatase staining and activity

ALP activity was localized in monolayer cultures by applying a diazonium substrate directly to the monolayers that was converted to a blue precipitate by the enzyme. In control cultures, diffuse and faint ALP activity was evident in both the BM and AD monolayers, but no stain development was evident in the SYN cultures. In osteogenic cultures, intense ALP activity was present within and immediately around the cell aggregates in BM cultures, whereas staining in the SYN and AD monolayers was far less intense and more diffusely distributed (**Figure 10-11**). There did not appear to be the same concentration of ALP activity within the cellular aggregates of SYN and ALP cultures as was seen in the osteogenic BM monolayers.

The transcriptional analyses of ALP mRNA expression were consistent with the enzymatic localization findings. There was little or no increase in ALP expression in control cultures over the 14-day culture interval. Under osteogenic conditions, BM-MSCs increased ALP mRNA expression by approximately 40- and 60-fold, at 7 and 14 days, respectively. In contrast, ALP up-regulation in SYN and AD cultures was modest. The four-fold increase in ALP expression by day 14 in SYN cultures and the ten-fold increase in AD cultures were not statistically significant and, comparatively, constituted less than 10% of the ALP levels detected in BM cultures (**Figure 12**).

The results of bulk ALP enzymatic activity, measured in mmol/L p-nitrophenol and normalized to DNA content, were somewhat different. Osteogenic BM-MSC cultures increased ALP activity significantly at both 7 (greater than five-fold increase) and 14 days (greater than ten-fold increase). Osteogenic AD cultures also significantly increased ALP activity by day 14; by approximately 15-fold. The differential ALP activities of day 14 osteogenic BM and AD cultures (2.6-fold more in BM cultures), were less impressive than the transcriptional differences. In SYN cultures, basal ALP activity was comparatively low and, even under osteogenic conditions, there were negligible changes in ALP activity (**Figure 13 to 19**).

#### Expression of osteogenic genes

Accepting that matrix mineralization and ALP up-regulation are the cardinal indices of osteogenic differentiation, changes in mRNA levels of several genes linked to the osteogenic phenotype were also investigated. Runx2 and Osterix are transcription factors required for osteogenic differentiation, while Osteonectin and Osteomodulin are secreted proteins expressed by cells in the osteoblastic lineage.

Quantitative PCR analyses of Cbfa1/Runx2 mRNA expression revealed that basal expression of this transcription factor was remarkably similar in the three cell types (**Figure 20**). As expected, with osteogenic stimulation, BM-MSCs significantly increased Runx2 expression five-fold and six-fold increases at days 7 and 14, respectively. In contrast, there was no significant increase in Runx2 expression in either SYN or AD cultures.

Osx mRNA expression profiles were very different in that basal expression was over ten times higher in BM cultures than in SYN or AD cultures, although these differences did not reach statistical significance (**Figure 21**). Osx was significantly up-regulated in BM cultures (7.5

fold increase at day 7, and five-fold increase at day 14), whereas no significant up-regulation occurred in the SYN and AD cultures.

These results are critical, since Runx2 and Osx transcriptional activities are mandatory for osteogenesis and consequent bone formation, as graphically demonstrated by murine gene deletion models.

Despite the established association of Osteonectin with the osteoblastic lineage, Osn mRNA levels were not significantly elevated by osteogenic medium in any of the three cell types. In fact, Osn expression in osteogenic cultures was less than the corresponding control cultures by day 14 in all three cell types (**Figure 22**). Osteomodulin expression profiles were similarly uninformative. Basal Osm expression was approximately two-fold higher in BM cell cultures, but there was no significant up-regulation of Osm mRNA in any cell types during osteogenic stimulation (**Figure 23**).

## CHAPTER 5

### DISCUSSION

The experiments in this study were carried out to test the hypothesis that “SYN-MSCs, BM-MSCs and AD-MSCs have the same capacity for *in vitro* osteogenic differentiation, under identical culture conditions.” Osteogenic differentiation was assessed in third passage cell populations using a panel of primary outcome measures focused on matrix mineralization (up-regulation of ALP expression and activity, along with matrix mineral staining using the Alizarin Red and Von Kossa protocols), and secondary criteria of osteogenic gene up-regulation, using QPCR. The results of the study overwhelmingly disproved the hypothesis. BMN-MSCs were clearly more able to undergo osteogenic differentiation than SYN- or AD-MSCs.

Under the ‘umbrella’ of the study’s hypothesis, three specific questions were addressed:

1. Was the expression of osteogenic markers and activities by SYN-MSCs, BM-MSCs and AD-MSCs under basal (non-induced) culture conditions equivalent?
2. Within each cell source (synovium, bone marrow and adipose tissue), was there statistically significant evidence of osteogenic differentiation under appropriate culture conditions?
3. What were the comparative osteogenic differentiation capacities of the three cell sources?

With respect to question 1, the expression and/or activities of the osteogenic markers in the Day 7 Control groups of the three cell types were compared. These comparisons were carried out to determine whether one or more of the putative progenitor populations was more basally ‘predisposed’ or ‘committed’ to the osteogenic lineage than the others, or whether monolayer expansion through two passages effectively ‘wipes the phenotypic memory’ of these disparate

cell populations. Osterix mRNA levels were around ten-fold higher in BM control cultures than was measured in SYN or AD cultures, but this difference was not significant, due to considerable inter-sample variability. Overall, the comparative analyses indicate that BM-MSCs do not express higher levels of osteogenic markers under basal culture conditions than other progenitor populations and cannot be considered to be ‘pre-committed’ to the osteogenic lineage.

The second question is focused on the qualitative aspect of phenotypic commitment, as is commonly performed to demonstrate tri-lineage potential of MSC isolates. We assessed the ability of each cell type to undergo osteogenesis, using statistically significant increases in expression or activity as criteria for differentiation, independent of any reference to other cell sources or a ‘target cell type’ control. As expected, bone marrow-derived MSCs were capable of robust and consistent osteogenic differentiation by all criteria used within the 14-day period of the experiments, apart from Osteonectin and Osteomodulin up-regulation (discussed below). AD-MSC cultures maintained under osteogenic conditions for 14 days exhibited focal matrix mineralization and ALP localization in the stained monolayer assays and significantly up-regulated ALP activity, but there were no other indications of osteogenic differentiation. The SYN-MSC monolayer also showed focal up-take of Alizarin Red and Von Kossa stains at day 14, along with a diffuse increase in ALP localization; however, by all quantitative assays, the SYN-MSCs did not undergo significant osteogenic differentiation.

The third question addressed the comparative osteogenic capacities of the three cell types, with BM-MSCs cast as the positive controls for the experiments. The monolayer expansion activities and morphological appearance of the proliferating monolayers were very

similar; however, as noted above, the BM-derived cell populations were significantly more capable of osteogenic differentiation within the 14-day experimental interval than either SYN-MSCs or AD-MSCs. In light of these results, the hypothesis is conclusively rejected. These findings are consistent with previous comparisons of the relative osteogenic capacities of bone marrow- and adipose-derived cells (Im, Shin et al. 2005; Kern, Eichler et al. 2006; Vidal, Kilroy et al. 2007). The failure of equine SYN-MSCs to express osteogenic phenotypic markers to any significant degree is not consistent with analogous experiments using synovial cells from rats and humans (Sakaguchi, Sekiya et al. 2005; Yoshimura, Muneta et al. 2007). However, in these studies, osteogenic monolayers were maintained for 21 days prior to phenotypic assessment.

There are several potential explanations for the relative inability of SYN- and AD-MSCs to undergo robust osteogenic differentiation in standard osteogenic media. It is possible that three monolayer passages were insufficient to generate sufficiently purified ‘stem cell’ populations from the initial mixed cell populations within synovial and adipose tissue digests. This possibility is supported by the appearance of the ‘osteogenic’ SYN and AD monolayers, where the characteristic multicellular osteogenic aggregates were few and far between. Previous studies in the Stewart lab using trabecular explant-derived cell populations indicate that the osteogenic capacity of these cells did increase with passage. Published studies {Estes et al 2006} and ongoing preliminary experiments in the Stewart lab suggest a similar effect of passage on the osteogenic capacity of adipose-derived cells, but this effect was not observed during early passage of synovium derived cells (**Figure 24**). Clearly, technologies to identify *bona fide* stem cell populations in the initial tissue digests, such as differential substrate adherence or surface epitope FACS separation would mitigate the need for extensive passage and accelerate the

generation of relatively pure MSC stocks. To date however, these strategies have not been developed for equine cell specimens (Stewart and Stewart 2011).

The osteogenic medium used in the differentiation cultures used FBS, dexamethasone, ascorbic acid and  $\beta$ -glycerophosphate to drive osteogenic differentiation. It is possible that additional osteogenic stimulation by exogenous BMP administration might induce more robust osteogenesis in SYN- and AD-MSCs, although BM-MSCs are clearly able to differentiate without this additional stimulus. Chondrogenesis studies using adipose-derived MSCs indicate that these cells require additional exposure to BMP-6, in addition to BMP-2, to affect full chondrogenesis (Estes, Wu et al. 2006). A similar BMP-mediated deficiency could exist in SYN-MSCs and AD-MSCs in an osteogenic context. Investigation of endogenous BMP induction under osteogenic conditions in these cell types would clarify this possibility.

Regardless of the specific reason(s) for the reduced osteogenic potential of equine SYN- and AD-MSCs, the molecular basis for this finding is clearly evident from the results of these experiments. Both Runx2 and Osterix up-regulation and transcriptional activity are required for osteogenesis, as has been compellingly demonstrated in murine gene deletion models (Komori, Yagi et al. 1997; Nakashima, Zhou et al. 2002; Zhou, Zhang et al. 2010). Neither Runx2 nor Osterix were significantly up-regulated in SYN- or AD-MSC cultures maintained in osteogenic medium. In particular, Osterix transcript levels were 100-fold lower in ‘osteogenic’ synovial and adipose cell groups than were measured in bone marrow cells. Inadequate BMP up-regulation in SYN and AD cells might be responsible for the failure of Runx2 and Osx induction, or it is also possible that the requisite promoter and enhancer sequences required for up-regulation of these genes are inaccessible in SYN- and AD-MSC chromatin. Regardless of the specific mechanism(s) that distinguishes the osteogenic potentials of bone marrow, synovial and adipose

progenitor populations, these results strongly support the position that stem cell populations from specific tissue and fluid sources retain source-specific lineage potentials and predispositions. This observation needs to be taken into account with any anticipated clinical application of MSC populations. Self-evidently, bone marrow-derived cells should be preferred for any application focused on bone regeneration.

The results of this study raise the obvious concern that the synovial cells isolated and expanded for these experiments and, to a slightly less extent, the adipose cells also, should not be considered 'stem cells' since they were unable to express osteogenic markers to any significant degree. In fact, there was sufficient stain up-take (ALP, Alizarin Red and Von Kossa) in the osteogenic monolayer cultures of both cell types that appropriately 'osteogenic' (though not exactly representative) aggregates could be documented; criteria of osteogenic induction that have been considered acceptable in many published studies.

Finally, the collective outcomes of these studies emphasize the need for multi-assay panels for rigorous assessments of MSC lineage commitment. Although ALP assays are straightforward and inexpensive protocols, ALP induction can occur in the absence of any other osteogenic transition. Further, the negligible changes in expression of Osteonectin and Osteomodulin mRNA levels in these experiments indicate that these gene targets are not informative for osteogenic induction studies. Additional lineage-specific gene targets will need to be identified for ongoing studies of equine osteogenesis.



## CHAPTER 6

### CONCLUSIONS

The experiments in this study were carried out to define the osteogenic potential of equine MSCs derived from bone marrow, adipose tissue and synovium, maintained under the same range and culture conditions.

The hypothesis that **“BM-, AD- and SYN-derived MSCs have the same *in vitro* osteogenic differentiation capacity”** was conclusively rejected. Although all MSC sources in this study exhibited some osteogenic ability, BM-MSCs hold the highest *in vitro* osteogenic potential as demonstrated by osteogenic gene expression, mineral deposition and ALP activity. Given that MSC-like populations reside in specific “niches” and based in our results, it is likely that BM serves as home for a population of quiescent mesenchymal stem cells with osteogenic capacity that would normally respond to injury through a process of reactivation.

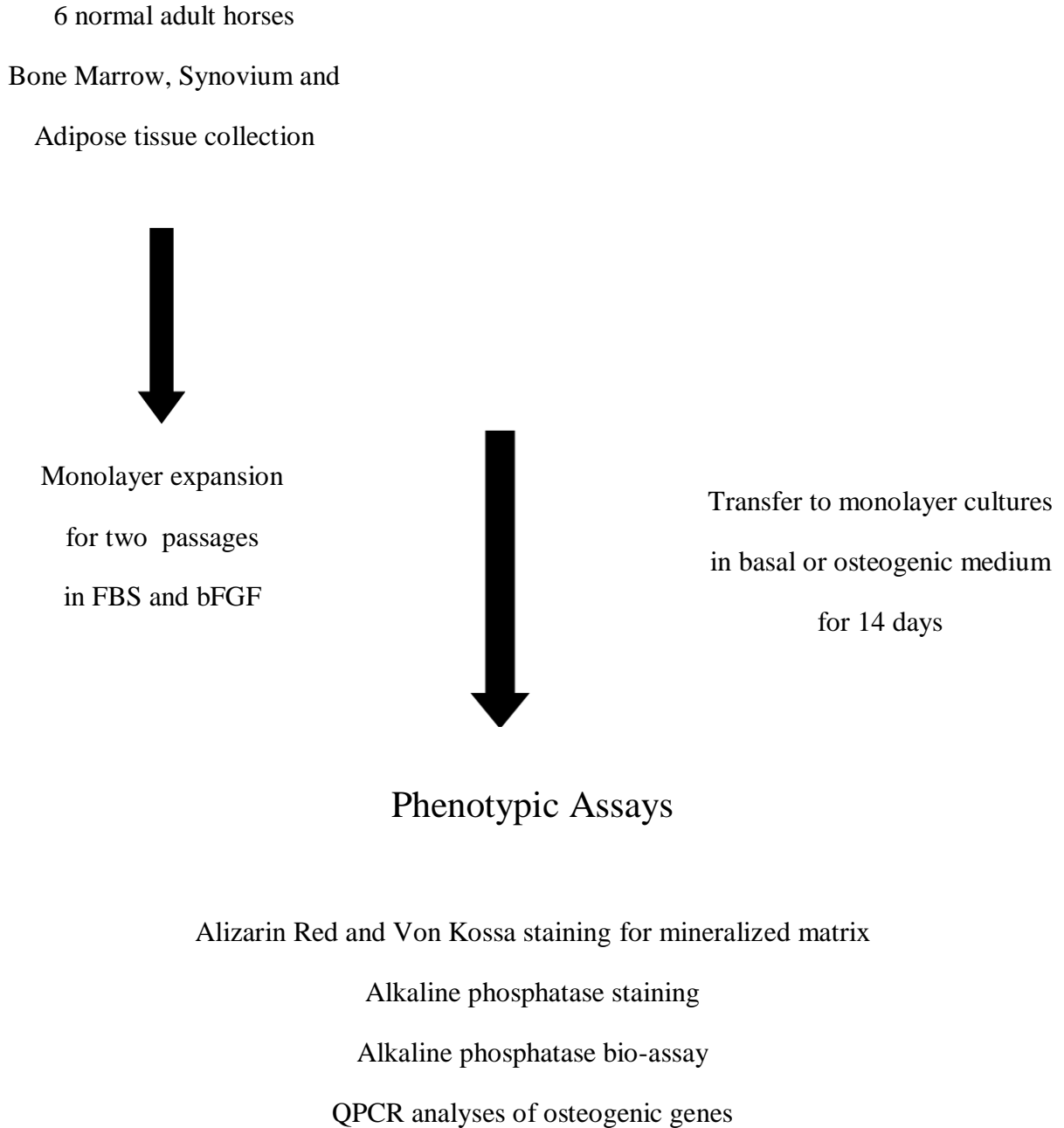
Future studies should be directed towards investigating the intrinsic up-regulation of osteogenic BMPs by SYN- and AD-MSC populations and assessing the effects of exogenous BMP administration on these cells. Further, the differential regulation of Runx2 and Osterix transcription in bone marrow-derived and other stem cell populations represents an excellent cellular model to study the molecular mechanisms by which source-specific progenitor populations retain lineage-targeted potential, despite extensive *in vitro* expansion and subsequent manipulation.

From a clinical perspective, strategies to utilize the considerable osteogenic capacities of bone marrow-derived MSCs should be developed and optimized to improve equine fracture

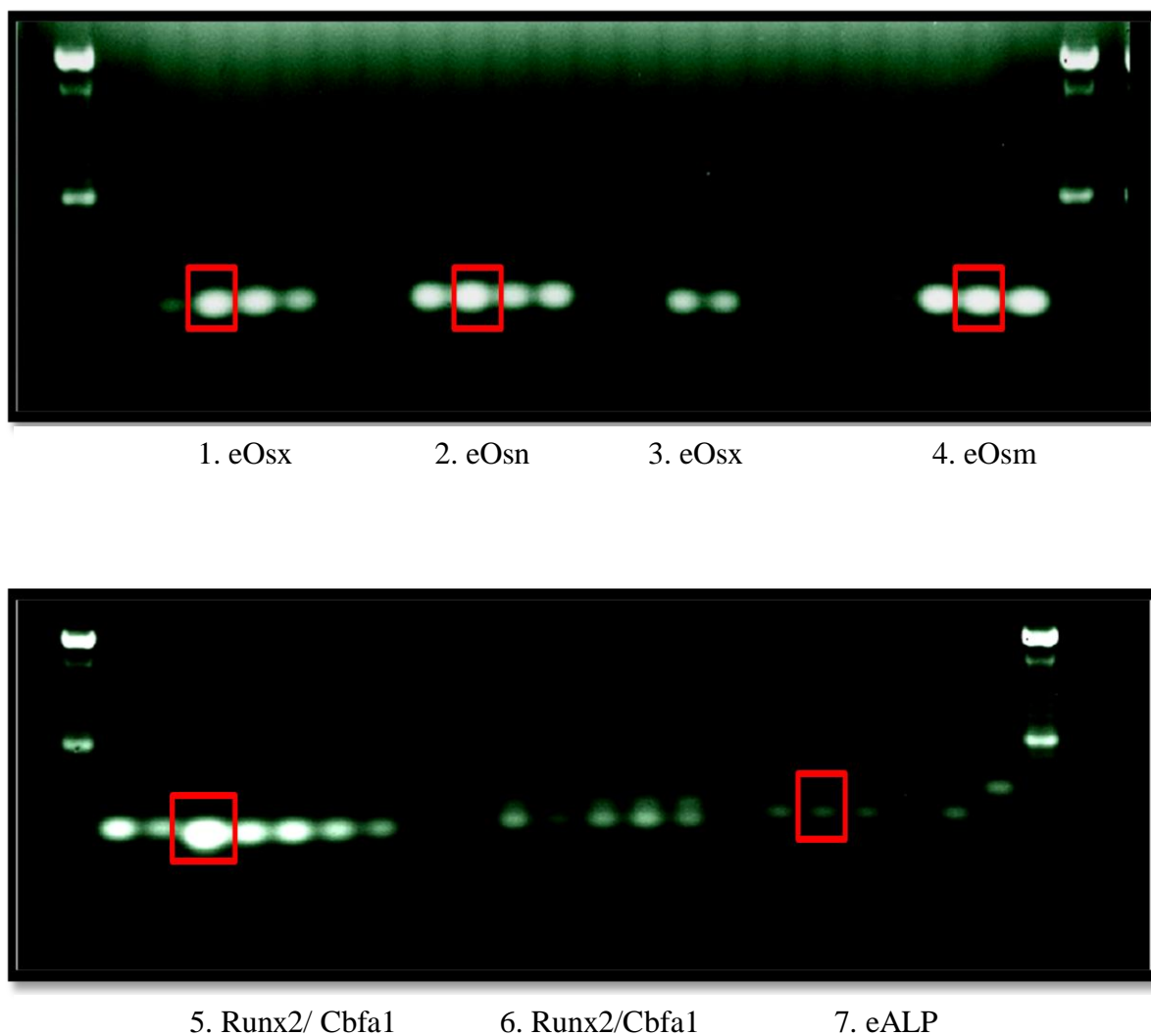
repair and subchondral defect repair/regeneration outcomes. In a more general context, feasible stem cell applications for clinical therapies will require considerable improvements in MSC identification, isolation and expansion to generate therapeutically useful numbers of cells with requisite activities in a timely manner. Conventional monolayer expansion requires at least 2 weeks and, even after several passages, does not necessarily generate multipotent cell populations, as the results of this study clearly demonstrate. Techniques for identifying and collecting progenitor cells on the basis of cell surface marker recognition show promise but an initial 'pure' population of stem cells still leaves the problem of rapid amplification while protecting the multipotent, immuno-modulatory, anti-inflammatory and/or trophic activities necessary for therapeutic efficacy.

## CHAPTER 7

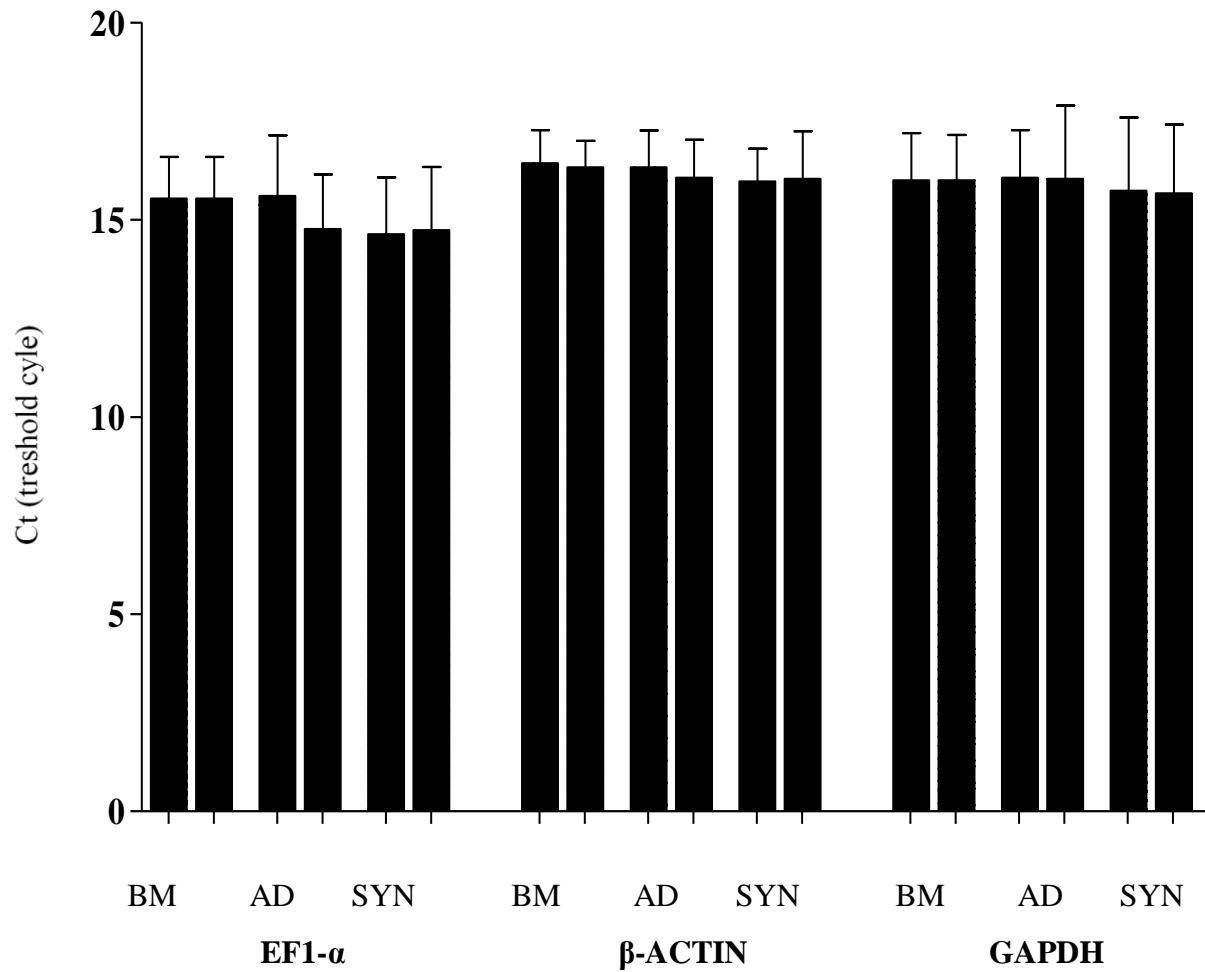
### FIGURES AND TABLES



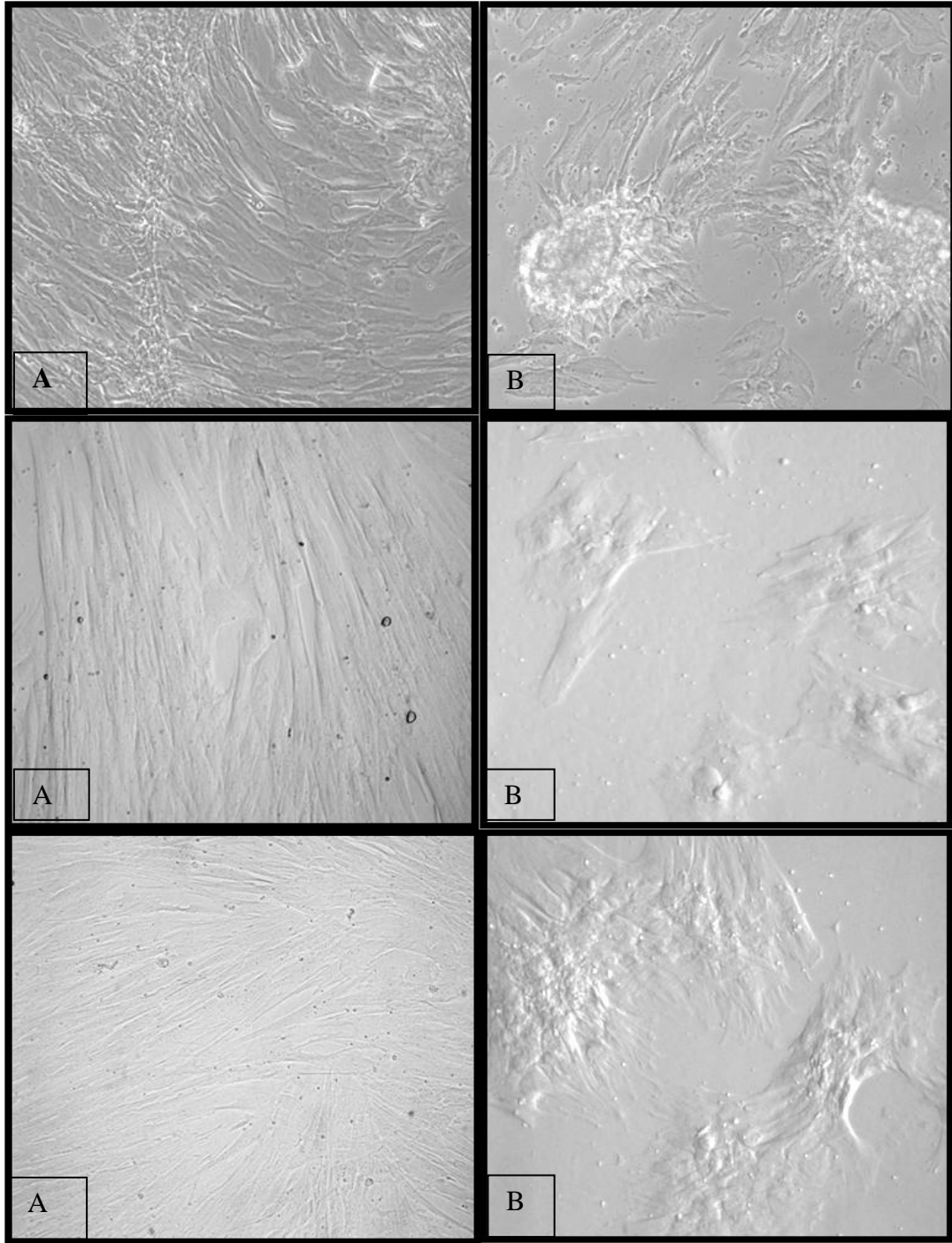
**Figure 1.** Flow chart of experimental methods



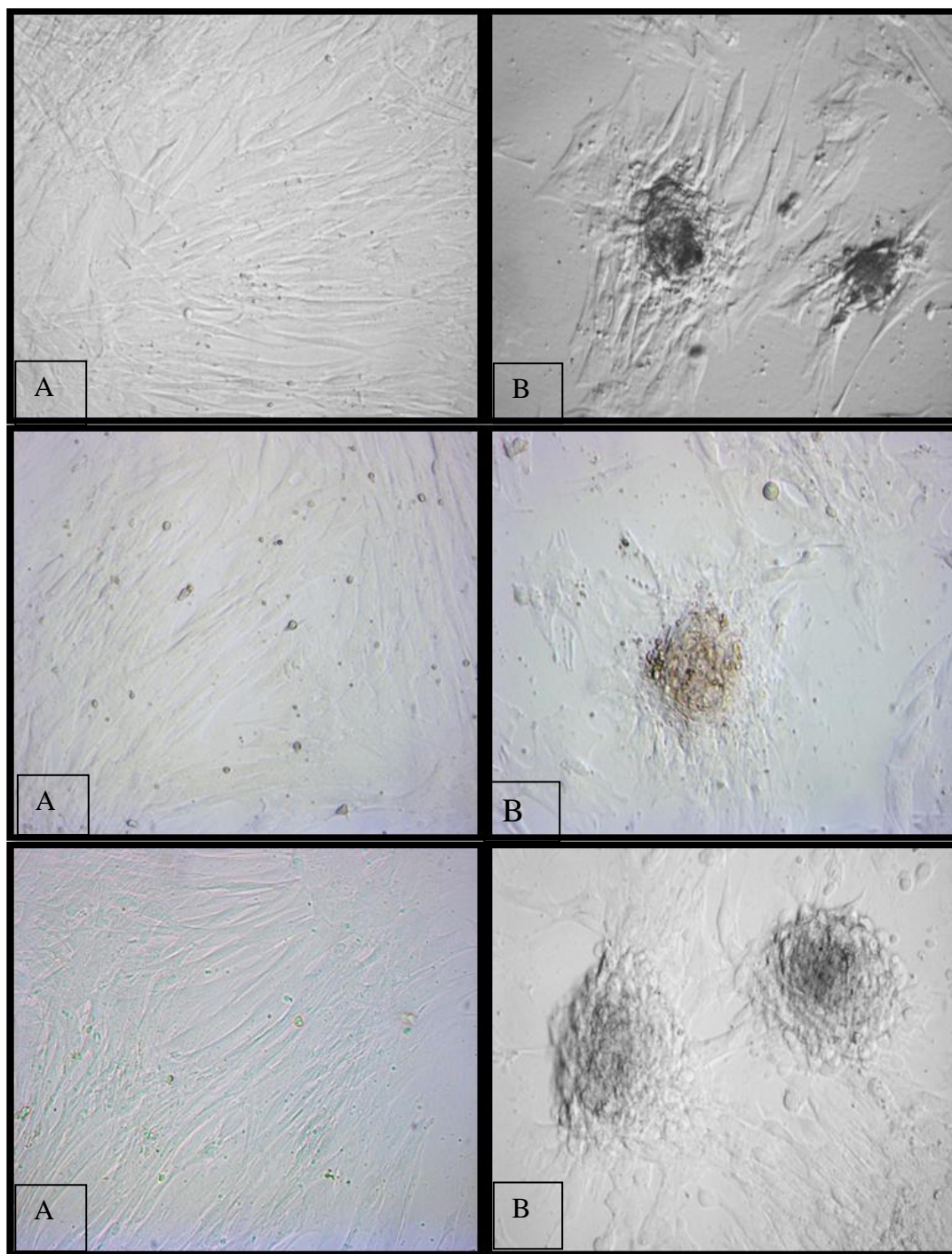
**Figure 2.** PCR primer optimization. The optimum annealing temperature of 57.7 °C for the designed primers targeting Osterix, Runx2, ALP, Osn, and Osm was determined by a gradient of annealing temperatures (54.8°C, 57.7°C, 60.3°C, 62.1°C and 64°C) . A 100 bp DNA ladder was included on each side of the gel to determine the size of the amplicon



**Figure 3.** Assessment of reference gene expression consistency. Expression profiles of the candidate reference genes elongation factor -1 alpha (EF1- $\alpha$ ),  $\beta$ -Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were assessed in several experiments. All three showed adequate consistency of expression.

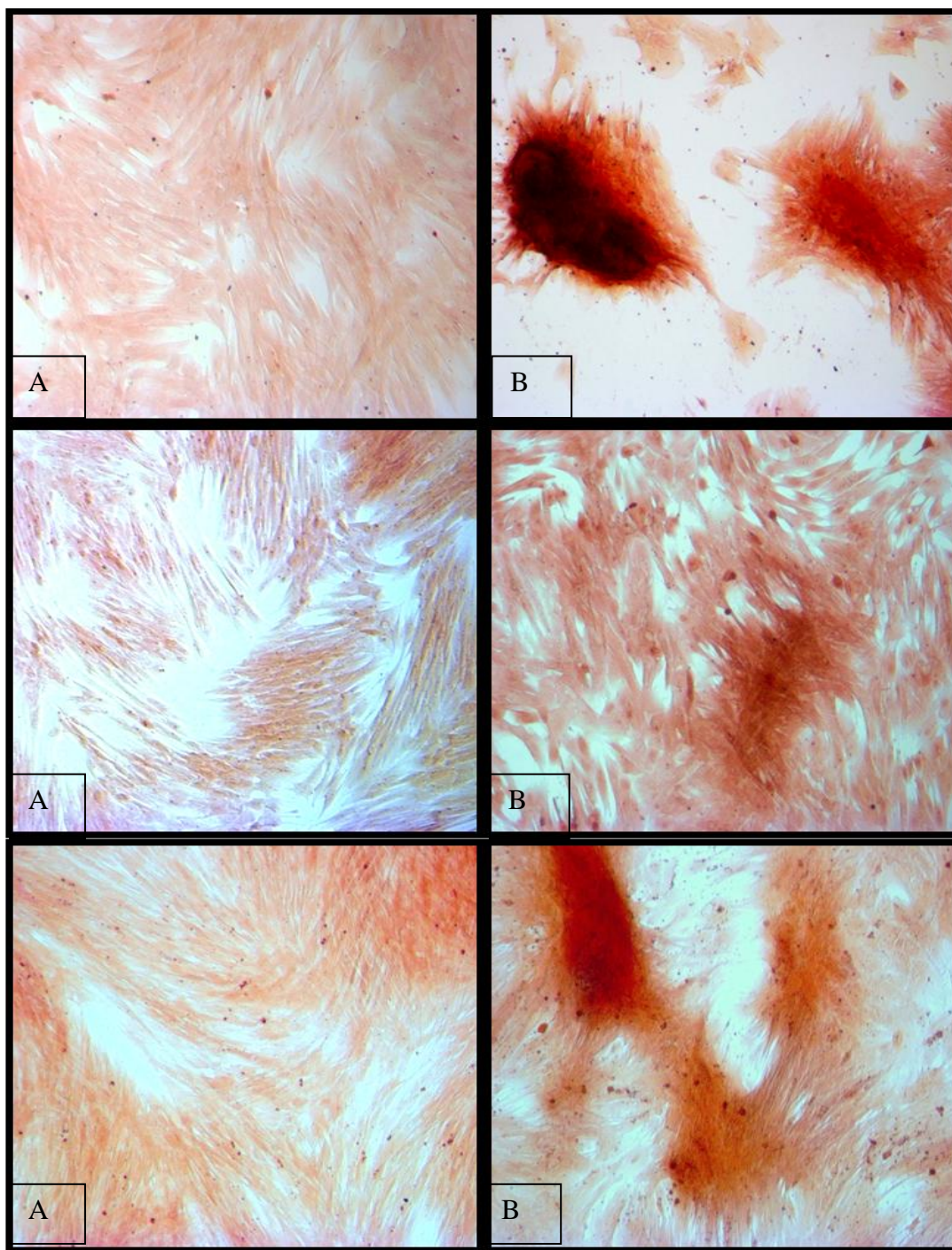


**Figure 4.** Contrast light microscopy (X 20 magnification) of Day 7 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.



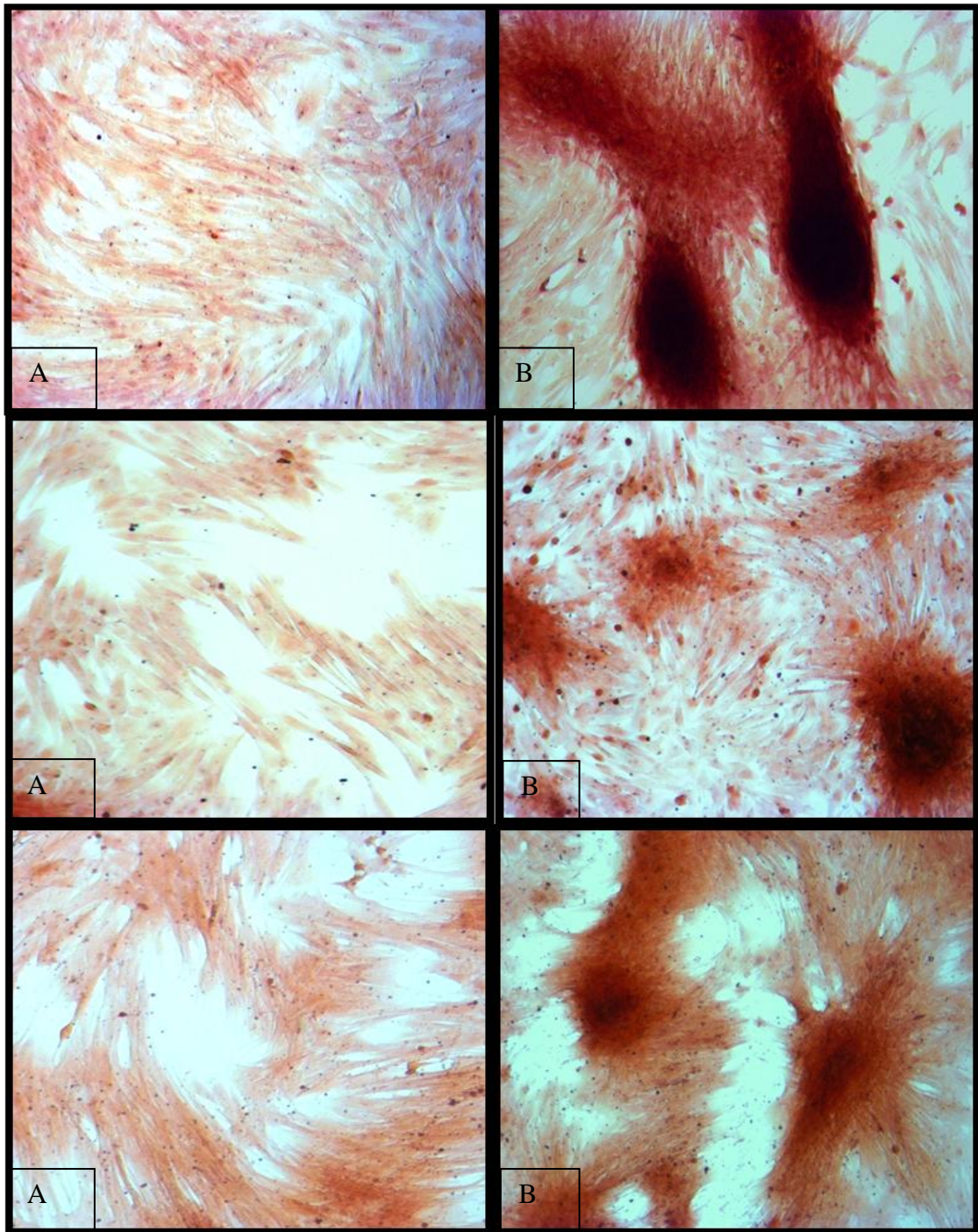
**Figure 5.** Contrast light microscopy (X 20 magnification) of Day 14 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.





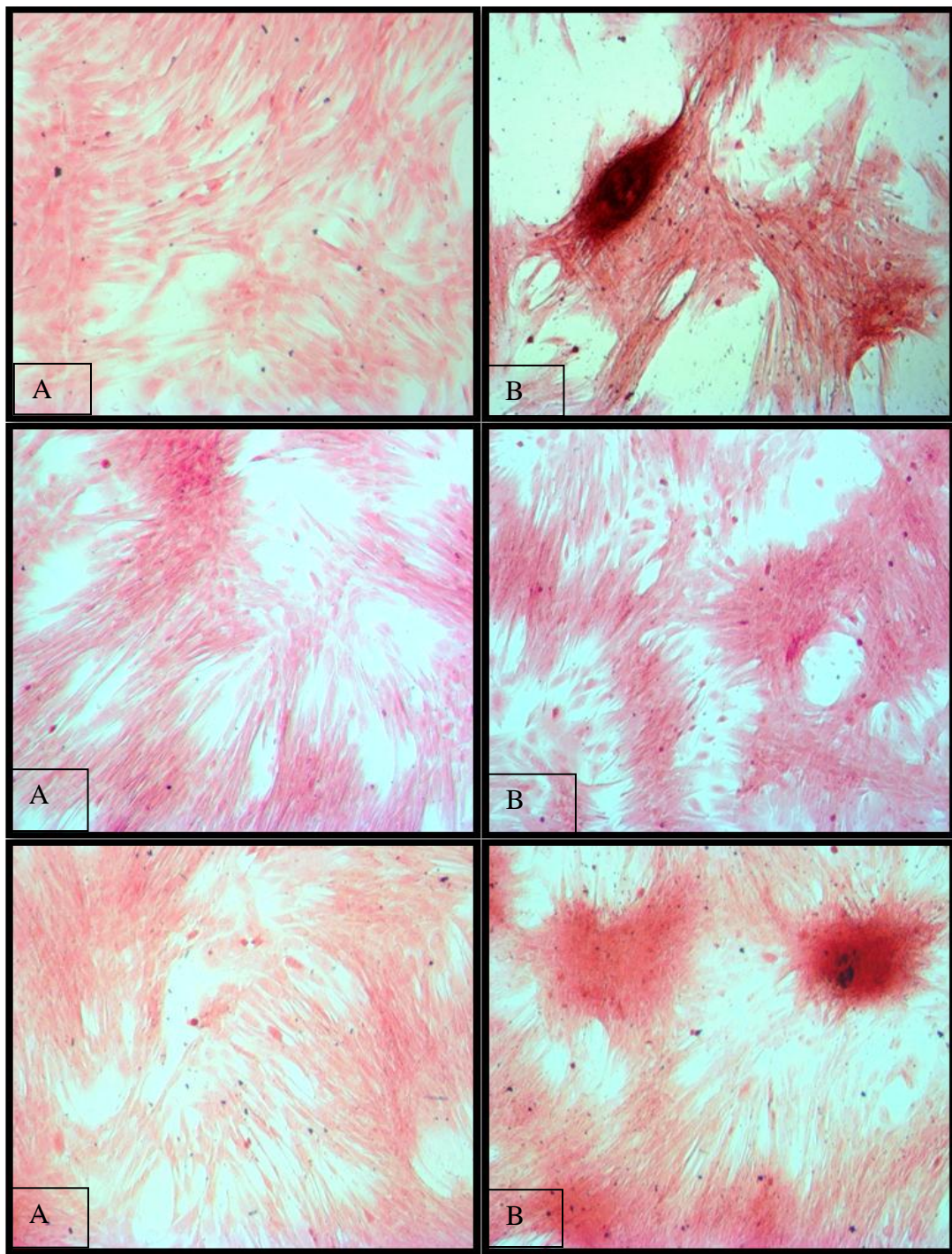
**Figure 6.** Alizarin Red staining (X 10 magnification) of Day 7 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.





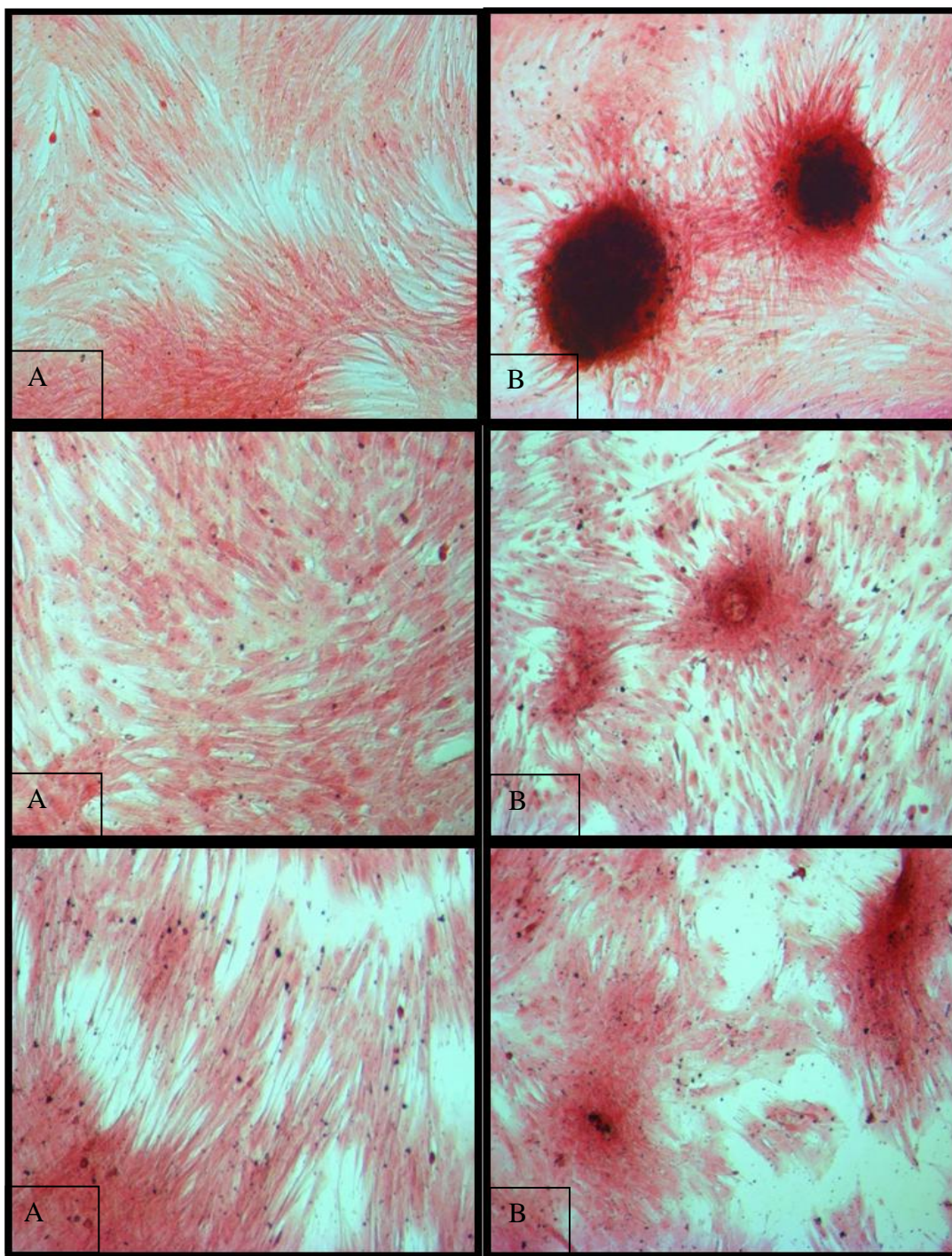
**Figure 7.** Alizarin Red staining (X 10 magnification) of Day 14 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.





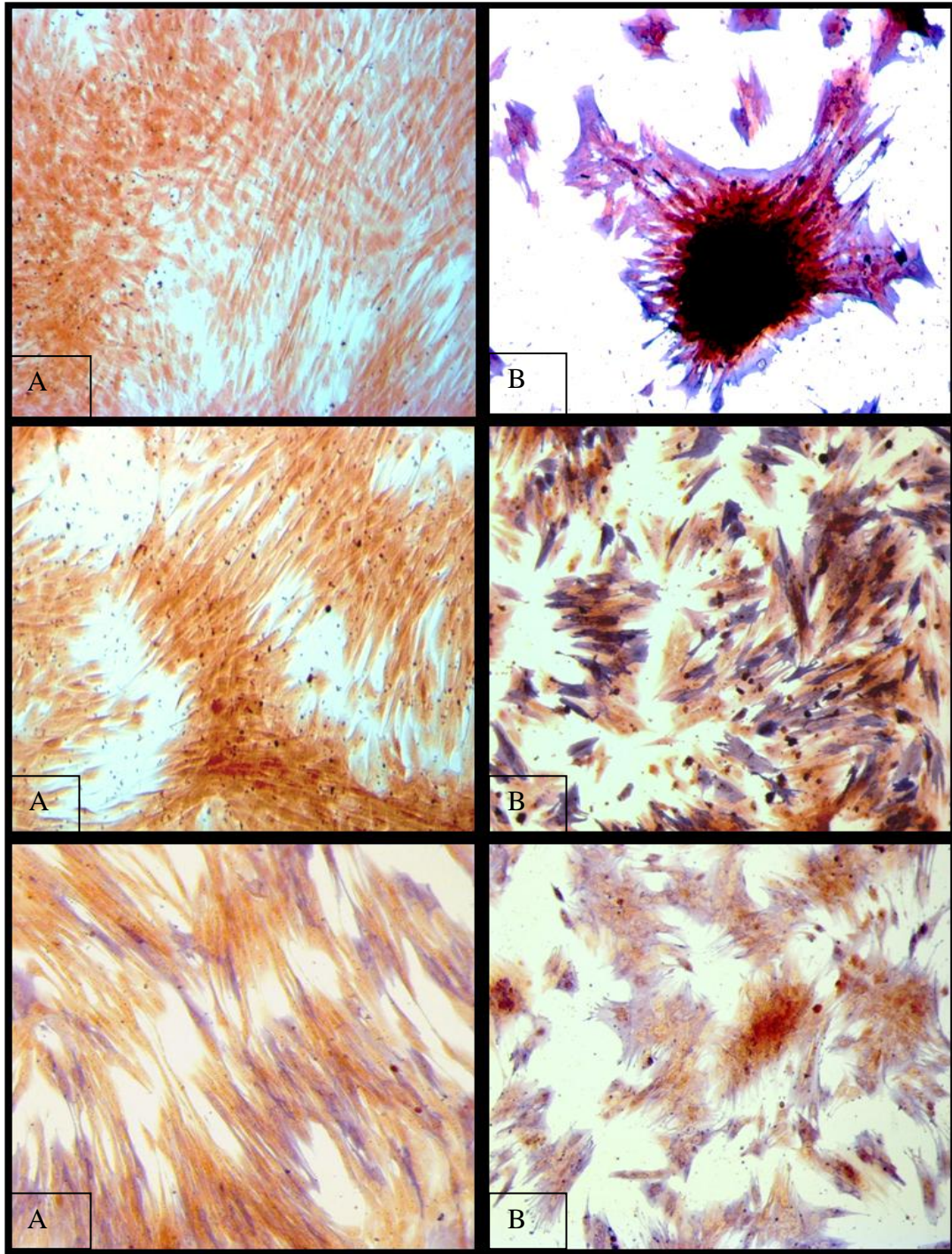
**Figure 8.** Von Kossa staining (X 10 magnification) of Day 7 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.





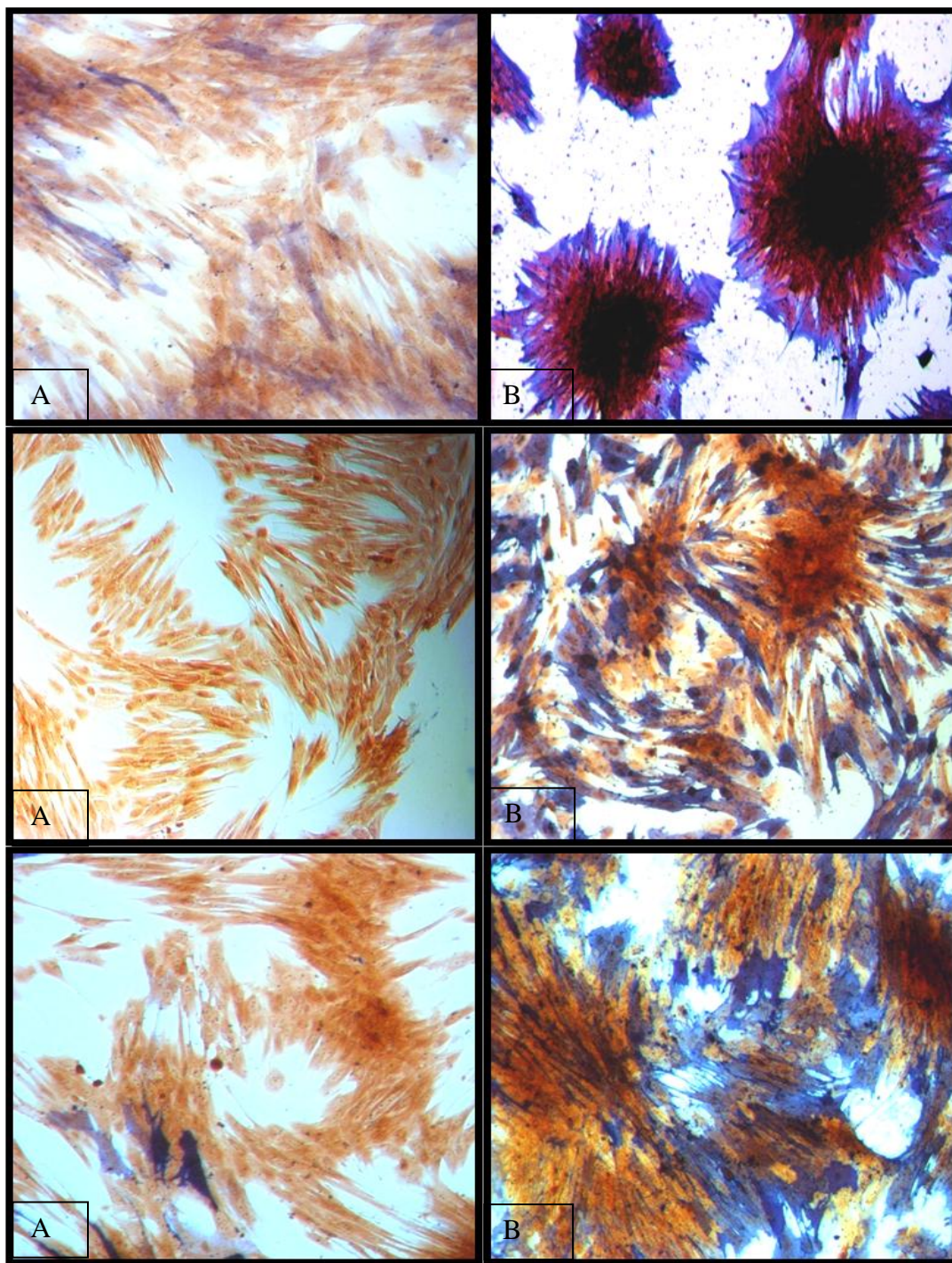
**Figure 9.** Von Kossa staining (X 10 magnification) of Day14 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.



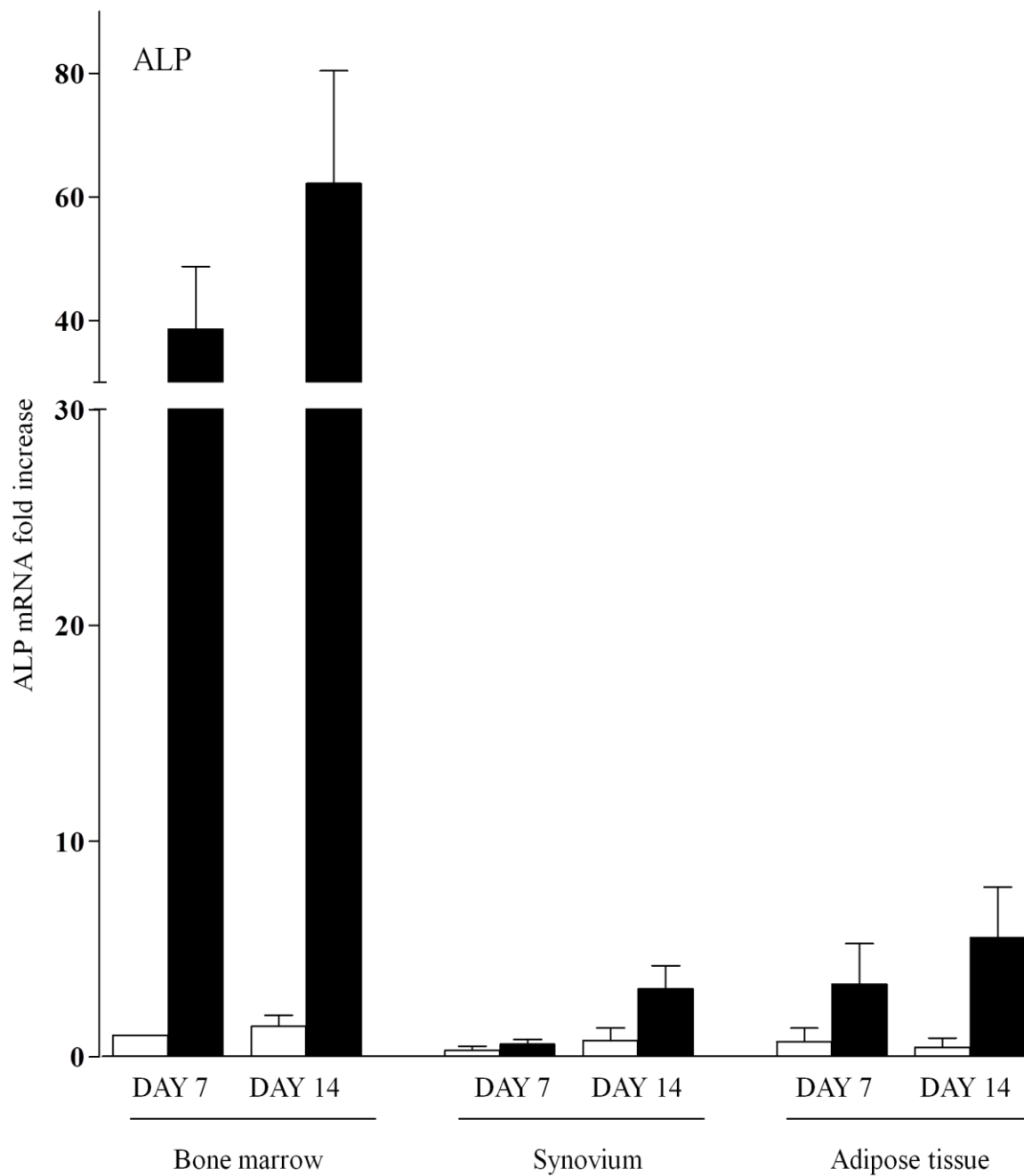


**Figure 10.** Alkaline Phosphatase staining (X 10 magnification) of Day 7 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.

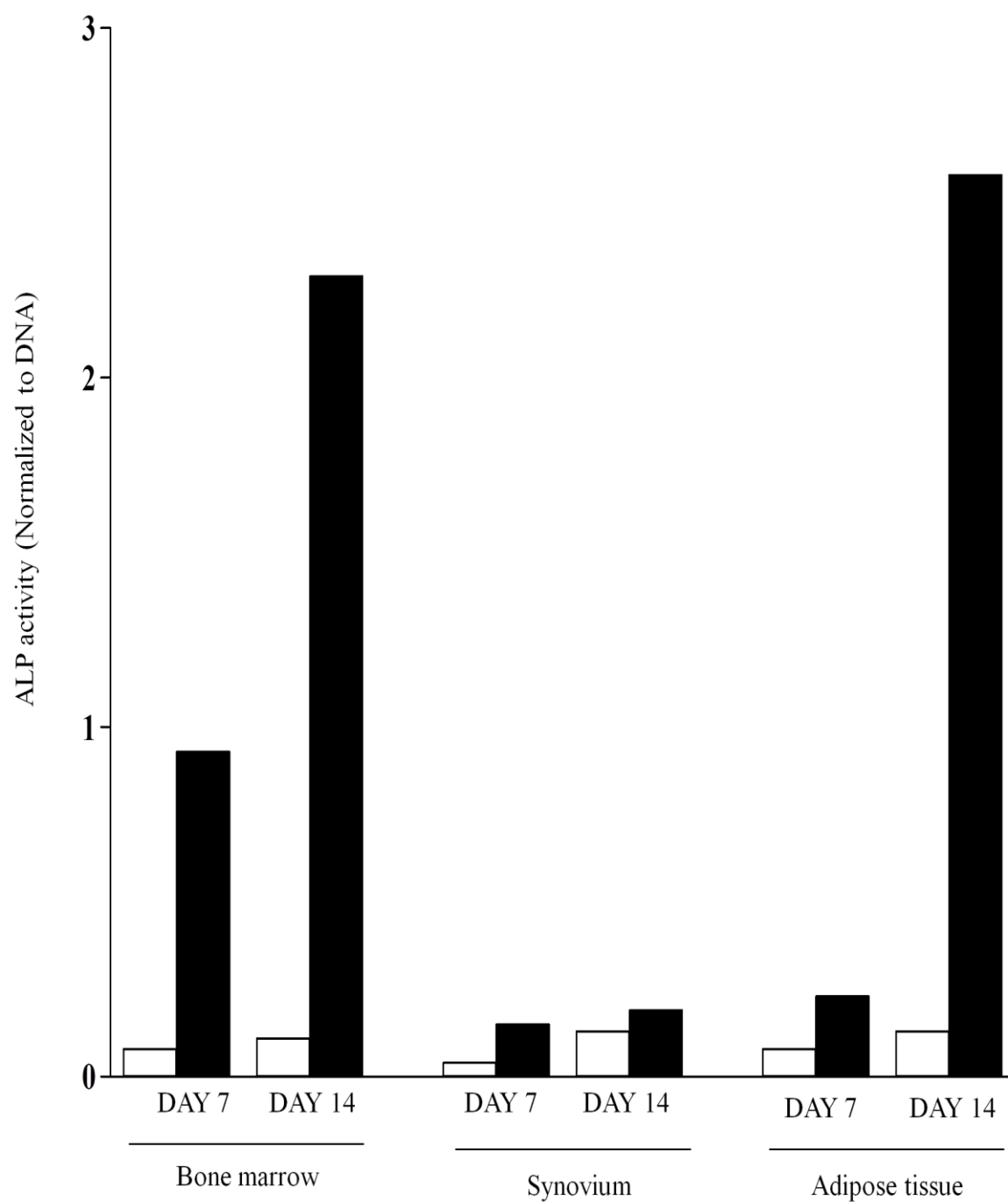




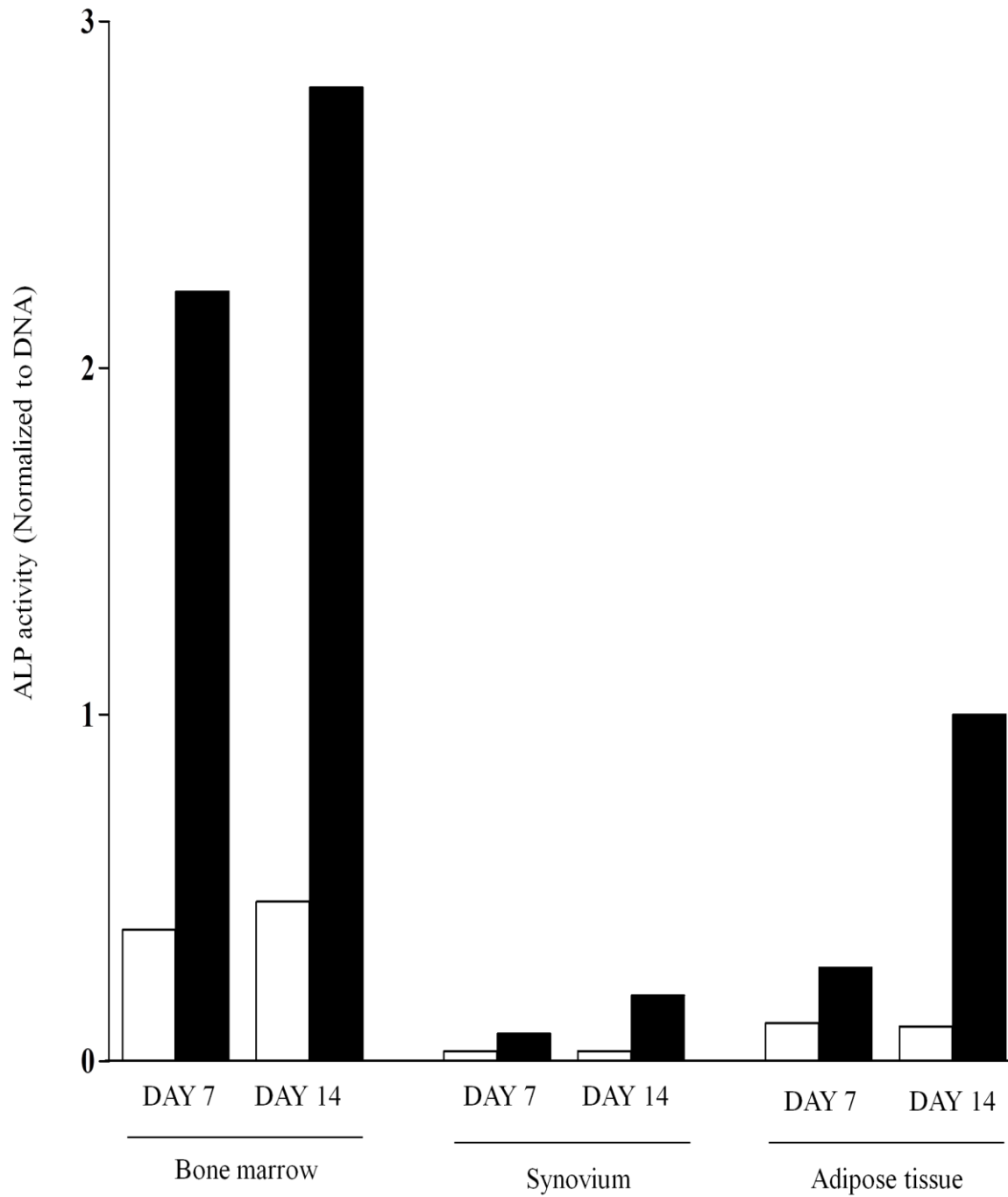
**Figure 11.** Alkaline Phosphatase staining (X 10 magnification) of Day 14 cultures. Control (**A**) and osteogenically induced (**B**) cells from BM- (upper panels), SYN- (middle panels) and AD- (lower panels) derived MSCs.



**Figure 12.** Grouped outcomes of six qPCR analyses of ALP mRNA fold change in BM-, SYN- and AD-derived MSCs cultures maintained in basal medium (white bars) or treated with osteogenic medium (black bars) for 14 days.

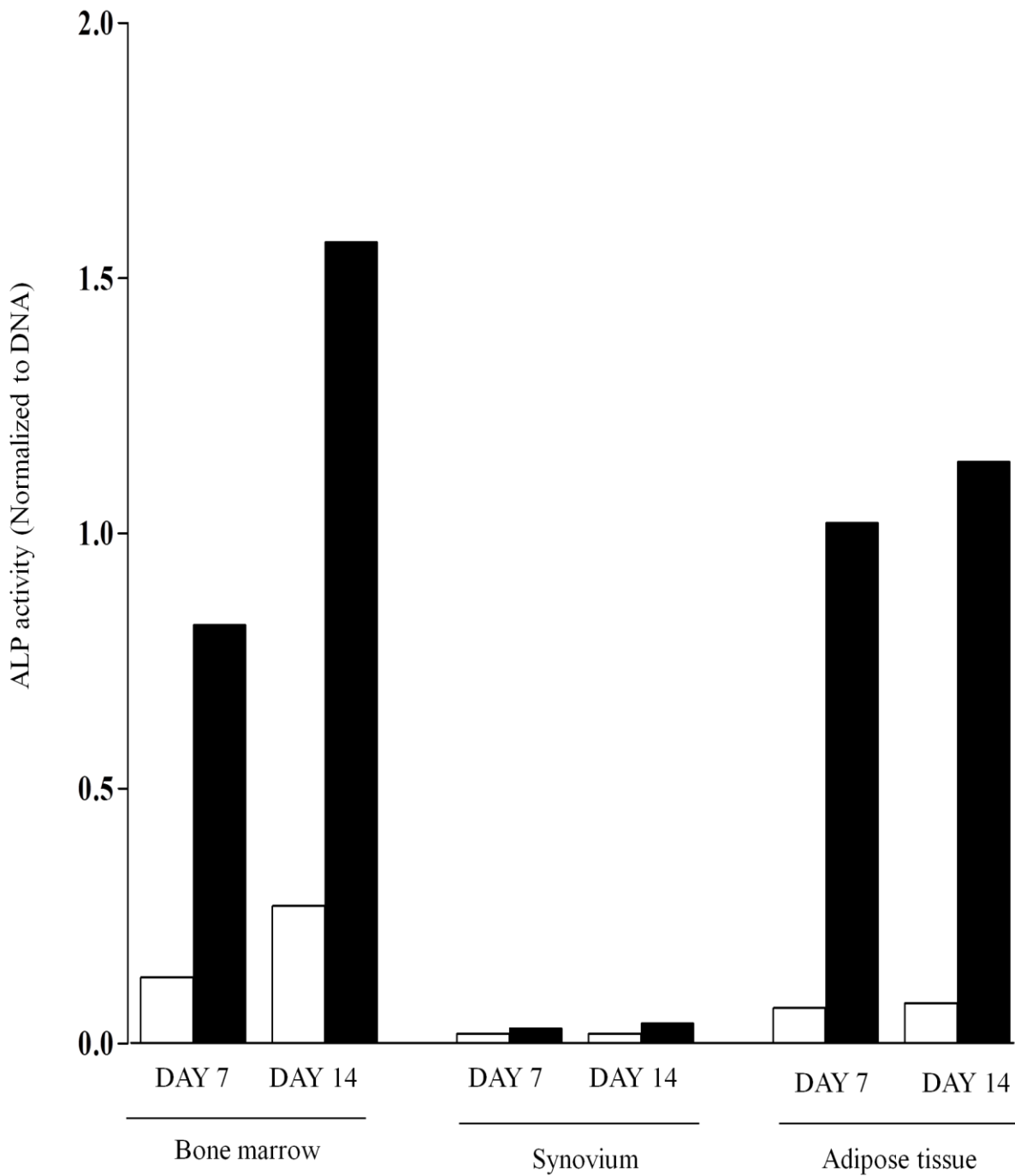


**Figure 13.** Horse 1. Changes in alkaline phosphatase (ALP) activity in cultured BM-, SYN- and AD-derived cells maintained in basal media (white bars) or treated with osteogenic media (black bars).

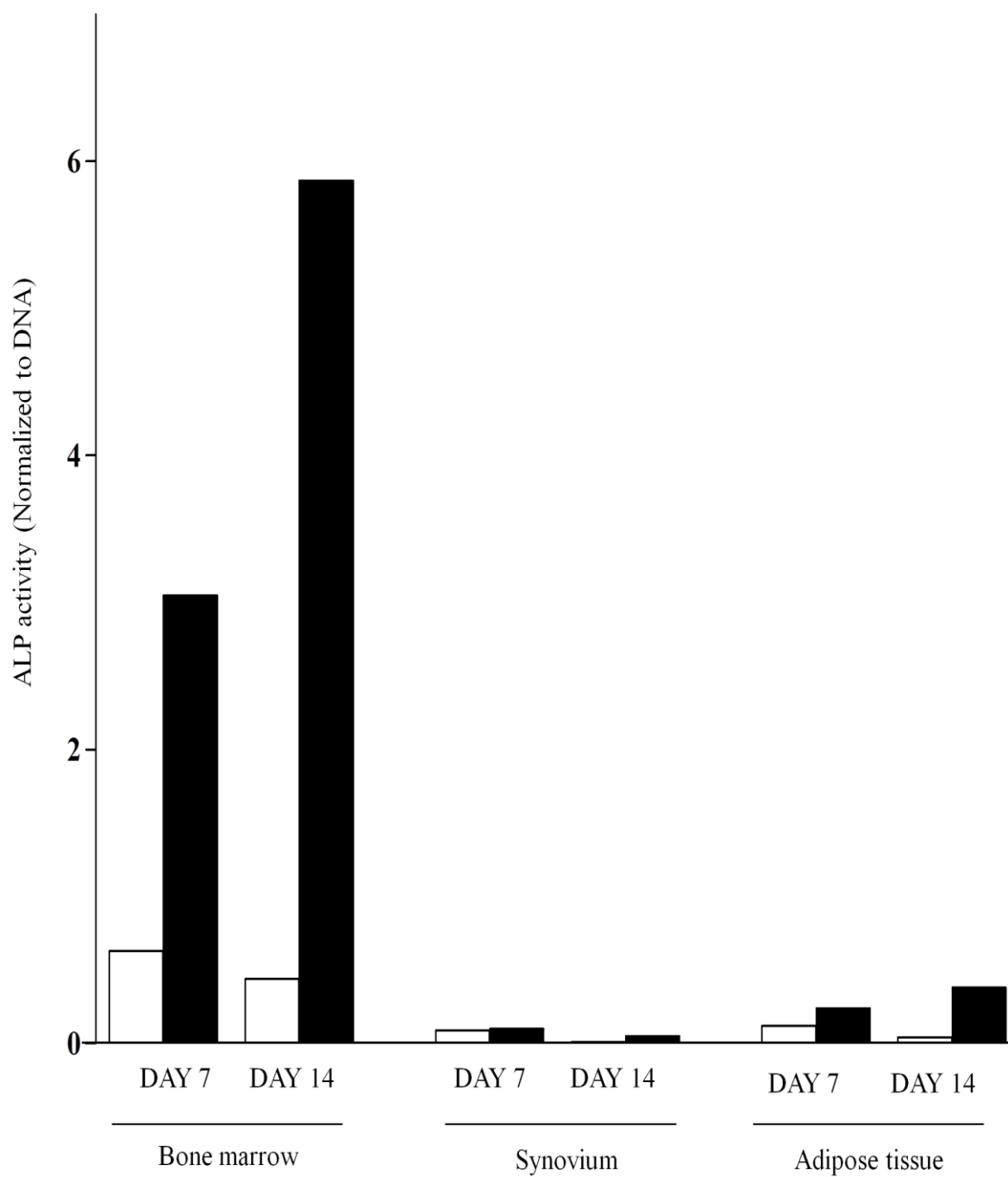


**Figure 14.** Horse 2. Changes in alkaline phosphatase (ALP) activity in cultured BM-, SYN- and AD cells maintained in basal media (white bars) or treated with osteogenic media (black bars).

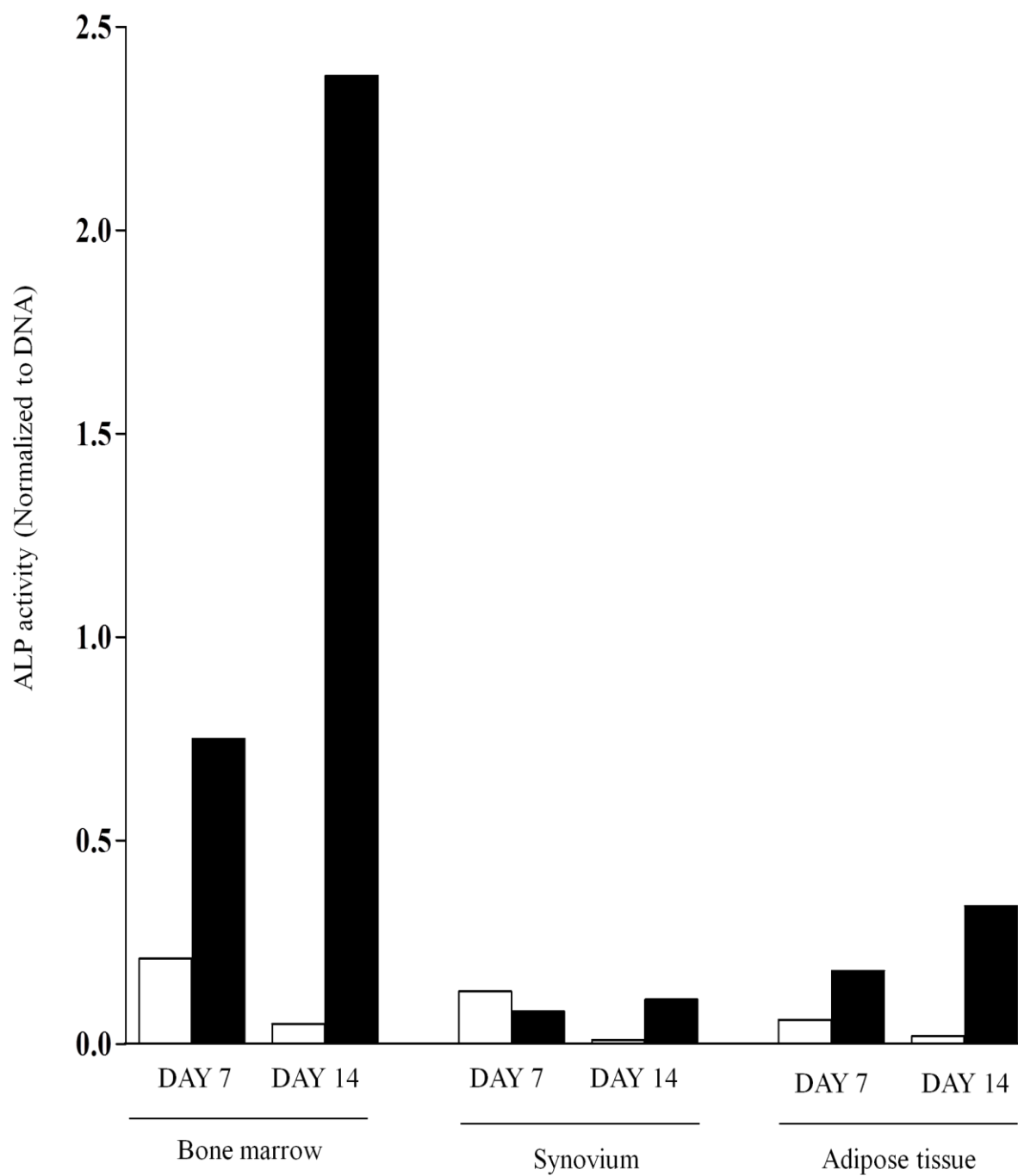




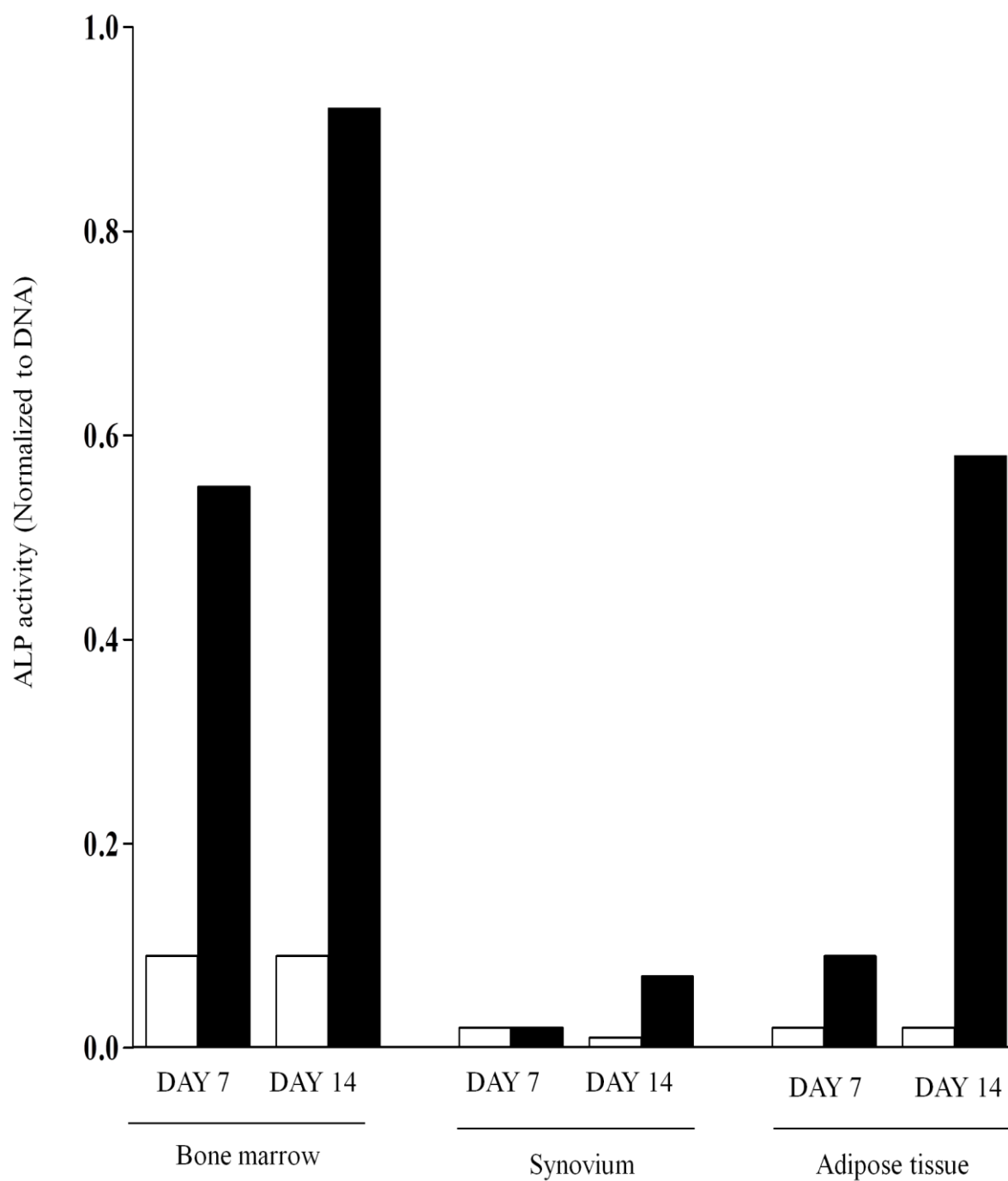
**Figure 15.** Horse 3. Changes in alkaline phosphatase (ALP) activity in cultured BM, SYN and AD cells maintained in basal media (white bars) or treated with osteogenic media (black bars).



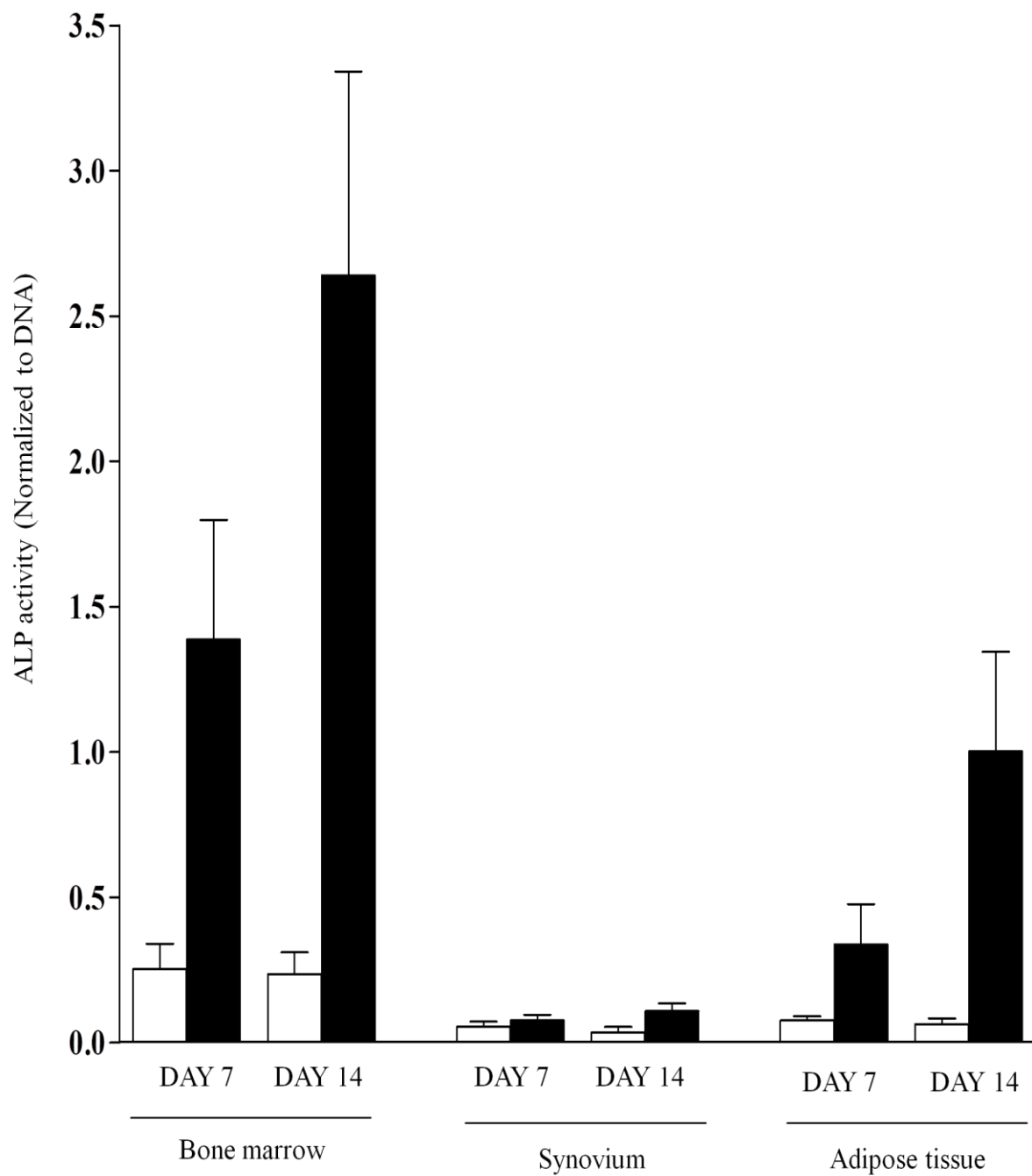
**Figure 16.** Horse 4. Changes in alkaline phosphatase (ALP) activity in cultured BM, SYN and AD cells maintained in basal media (white bars) or treated with osteogenic media (black bars).



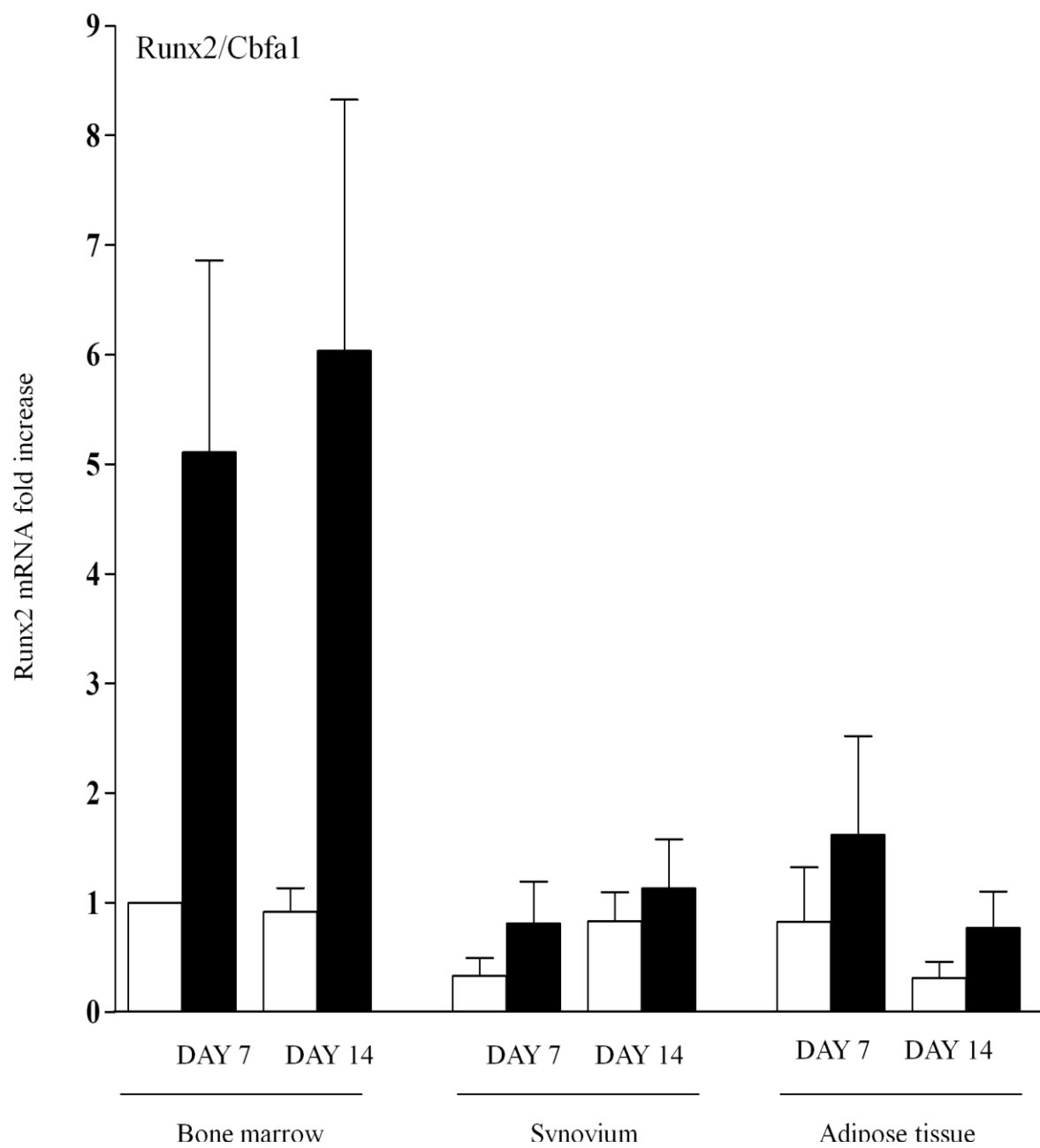
**Figure 17.** Horse 5. Changes in alkaline phosphatase (ALP) activity in cultured BM, SYN and AD cells maintained in basal media (white bars) or treated with osteogenic media (black bars).



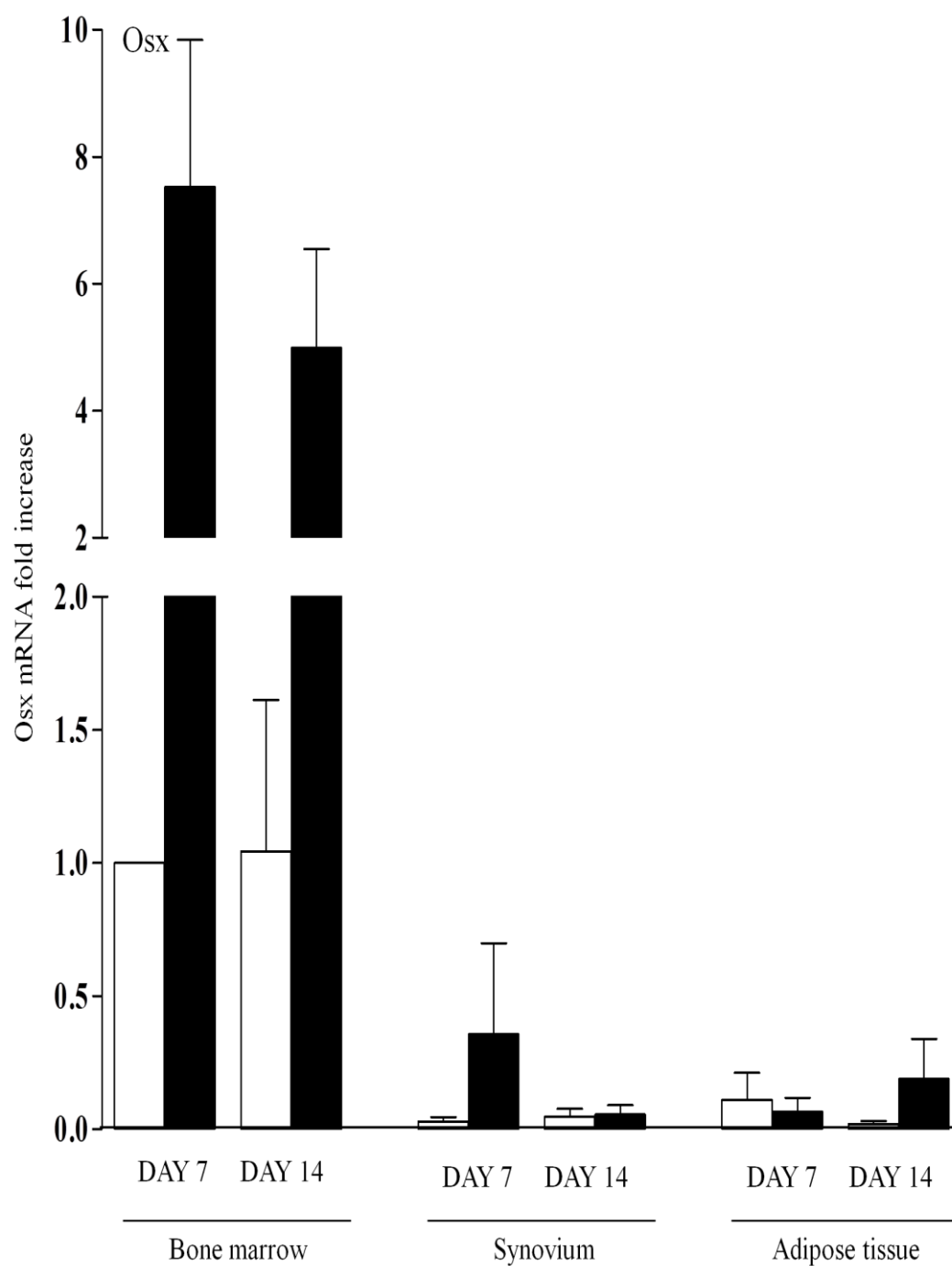
**Figure 18.** Horse 6. Changes in alkaline phosphatase (ALP) activity in cultured BM, SYN and AD cells maintained in basal media (white bars) or treated with osteogenic media (black bars).



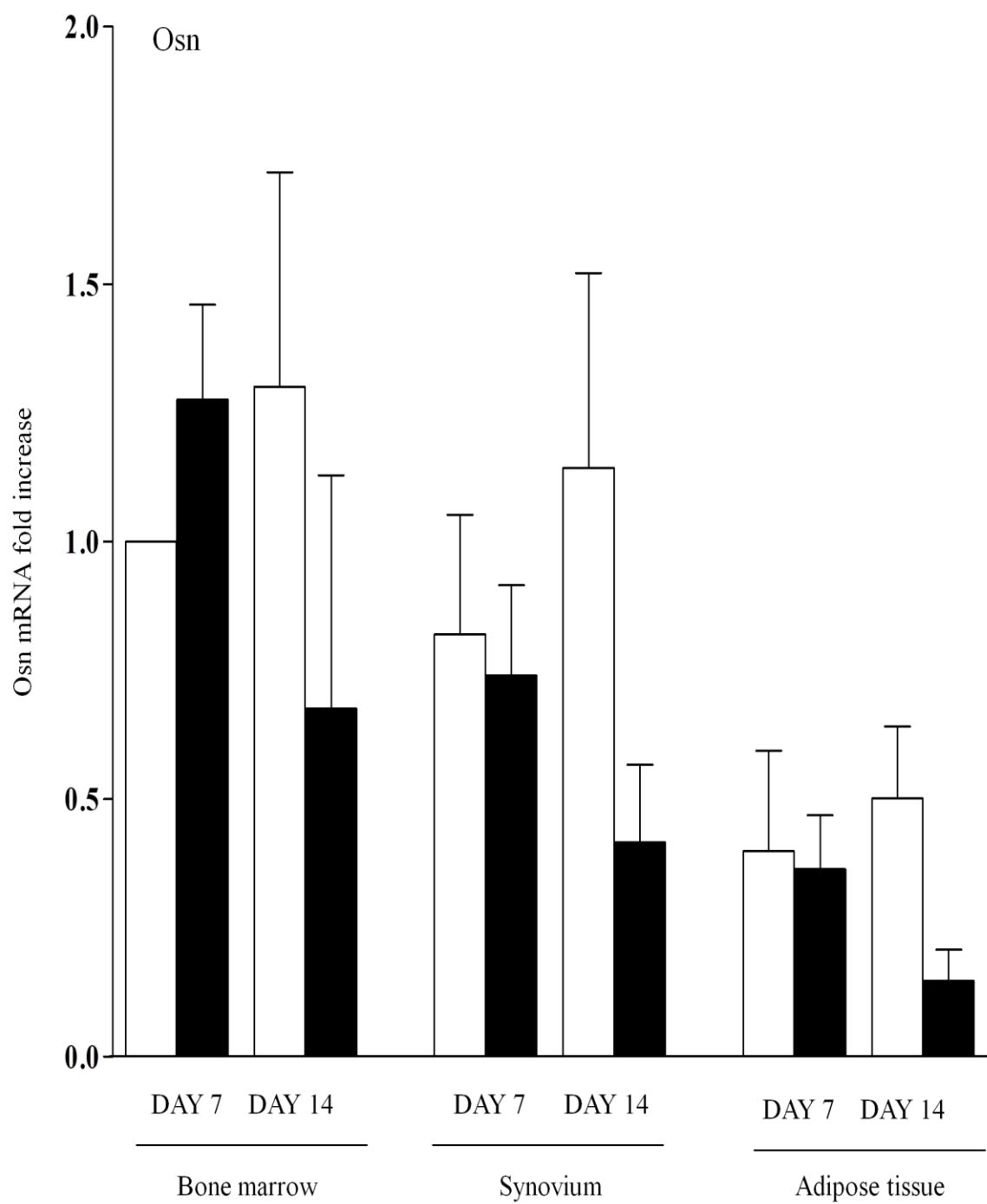
**Figure 19** (N=6 horses). Changes in alkaline phosphatase (ALP) activity in cultured BM, SYN and AD cells maintained in basal media (white bars) or in osteogenic media (black bars).



**Figure 20.** Grouped outcomes of six qPCR analyses of Runx2 mRNA levels in BM, SYN and AD cells maintained in basal medium (white bars) or osteogenic medium (black bars) for 14 days.

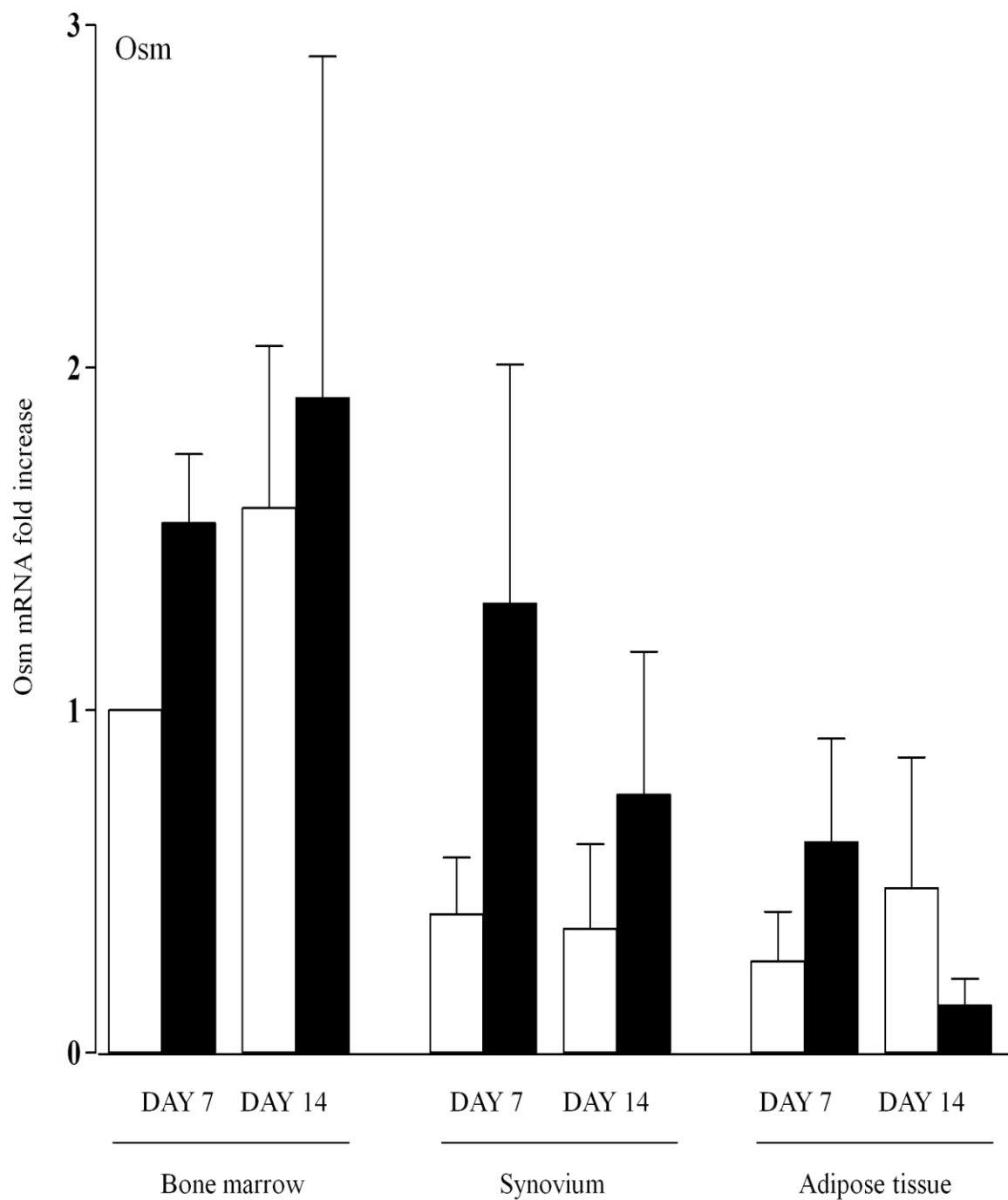


**Figure 21.** Grouped outcomes of six qPCR analyses of Osx mRNA levels in BM, SYN and AD cells maintained in basal medium (white bars) or osteogenic medium (black bars) for 14 days.

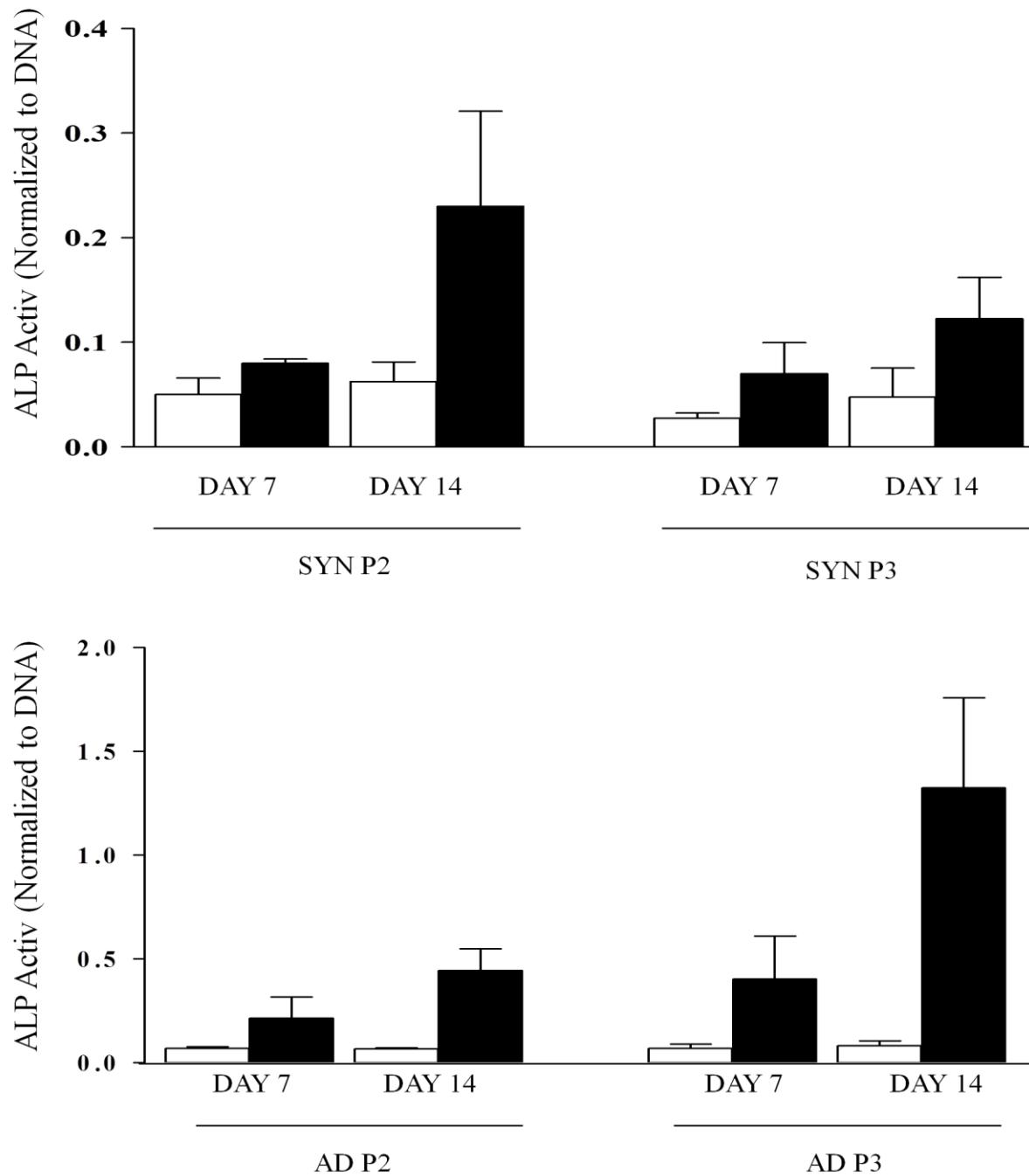


**Figure 22.** Grouped outcomes of six qPCR analyses of Osn mRNA levels in BM, SYN and AD cells maintained in basal medium (white bars) or osteogenic medium (black bars) for 14 days.





**Figure 23.** Grouped outcomes of six qPCR analyses of Osm mRNA levels in BM, SYN and AD cells maintained in basal medium (white bars) or osteogenic medium (black bars) for 14 days.



**Figure 24.** (N=4 horses) Effect of passage (P2 to P3) on ALP activity from SYN- (upper panel) and AD- (lower panel) derived MSCs cultured in basal medium (white bars) or osteogenic medium (black bars).

**Table 1:** Primers utilized in the qPCR reactions

Gene	Sense Primer	Annealing
size	Antisense Primer	temperature
RUNX2 (177 bp)	5' CAGACCAGCAGCACTCCATA (1315) 5' CAGCGTCAACACCATCATTC (1492)	57.7 °C
Osterix (207 bp)	5' GGCTATGCCAATGACTACCC 5' GGTGAGATGCCTGCATGGA	57.7 °C
ALP (221 bp)	5' TGGGGTGAAGGCTAATGAGG (357) 5' GGCATCTCGTTGTCCGAGTA (578)	57.7 °C
Osteopontin (190 bp)	5' AACCTTCTGACCGAGAAGCA (591) 5' TGGGACAGGTACCCATCAAT (781)	57.7 °C
Osteomodulin (199 bp)	5' TACATCCGTGTGGACCAAAA (1000) 5' TCTGGGCCTTCATGAGAATC (1199)	57.7 °C
EF- 1alpha (328 bp)	5' CCCGGACACAGAGACTTCAT (48) 5' AGCATGTTGTCACCATTCCA (376)	57.7 °C

**Table 2:** Comparative activities and expression of osteogenic indicators under basal conditions

Synovium control vs Bone Marrow control

	SYN Control Mean $\pm$ SEM	BM Control Mean $\pm$ SEM	P-VALUE	SIGNIFICANCE P-VALUE <0.05
ALP bio-activity at day 7	0.05 $\pm$ 0.01	0.25 $\pm$ 0.08	0.59	No
ALP bio-activity at Day 14	0.03 $\pm$ 0.01	0.23 $\pm$ 0.07	0.58	No
ALP mRNA levels at day 7	0.31 $\pm$ 0.17	1 $\pm$ 0	0.92	No
ALP mRNA levels at day 14	0.75 $\pm$ 0.59	1.42 $\pm$ 0.51	0.86	No
RUNX2 mRNA levels at day 7	0.33 $\pm$ 0.15	1 $\pm$ 0	0.86	No
RUNX2 mRNA levels at day 14	0.82 $\pm$ 0.26	0.91 $\pm$ 0.21	0.93	No
Osx mRNA levels at day 7	0.02 $\pm$ 0.01	1 $\pm$ 0	0.97	No
Osx mRNA levels at day 14	0.04 $\pm$ 0.03	1.04 $\pm$ 0.56	0.44	No
Osm mRNA levels at day 7	0.4 $\pm$ 0.16	1 $\pm$ 0	0.29	No
Osm mRNA levels at day 14	0.36 $\pm$ 0.24	1.59 $\pm$ 0.47	0.03	YES
Osn mRNA levels at day 7	0.82 $\pm$ 0.23	1 $\pm$ 0	0.57	No
Osn mRNA levels at day 14	1.14 $\pm$ 0.37	1.3 $\pm$ 0.41	0.61	No

Adipose control vs Bone Marrow control

	AD Control Mean $\pm$ SEM	BM Control Mean $\pm$ SEM	P-VALUE	SIGNIFICANCE P-VALUE <0.05
ALP bio-activity at day 7	0.07 $\pm$ 0.01	0.25 $\pm$ 0.08	0.63	No
ALP bio-activity at Day 14	0.06 $\pm$ 0.01	0.23 $\pm$ 0.07	0.64	No
ALP mRNA levels at day 7	0.7 $\pm$ 0.63	1 $\pm$ 0	0.99	No
ALP mRNA levels at day 14	0.45 $\pm$ 0.42	1.42 $\pm$ 0.51	0.92	No
RUNX2 mRNA levels at day 7	0.86 $\pm$ 0.48	1 $\pm$ 0	0.90	No
RUNX2 mRNA levels at day 14	0.31 $\pm$ 0.14	0.91 $\pm$ 0.21	0.59	No
Osx mRNA levels at day 7	0.11 $\pm$ 0.10	1 $\pm$ 0	0.41	No
Osx mRNA levels at day 14	0.02 $\pm$ 0.01	1.04 $\pm$ 0.56	0.43	No
Osm mRNA levels at day 7	0.26 $\pm$ 0.14	1 $\pm$ 0	0.19	No
Osm mRNA levels at day 14	0.48 $\pm$ 0.38	1.59 $\pm$ 0.47	0.05	No
Osn mRNA levels at day 7	0.39 $\pm$ 0.19	1 $\pm$ 0	0.06	No
Osn mRNA levels at day 14	0.5 $\pm$ 0.14	1.3 $\pm$ 0.41	0.01	YES

**Table 3:** Within-tissue analyses of osteogenic differentiation**BONE MARROW**

Bone marrow OI vs Bone marrow control

	BM OI Mean $\pm$ SEM	BM Control Mean $\pm$ SEM	P-VALUE	SIGNIFICANCE P-VALUE <0.05
ALP bio-activity at day 7	1.38 $\pm$ 0.41	0.25 $\pm$ 0.08	0.004	YES
ALP bio-activity at Day 14	2.64 $\pm$ 0.7	0.23 $\pm$ 0.07	0.004	YES
ALP mRNA levels at day 7	38.62 $\pm$ 10.11	1 $\pm$ 0	< 0.001	YES
ALP mRNA levels at day 14	62.18 $\pm$ 18.25	1.42 $\pm$ 0.51	< 0.001	YES
RUNX2 mRNA levels at day 7	5.10 $\pm$ 1.75	1 $\pm$ 0	< 0.001	YES
RUNX2 mRNA levels at day 14	6.03 $\pm$ 2.29	0.91 $\pm$ 0.21	< 0.001	YES
Osx mRNA levels at day 7	7.52 $\pm$ 2.31	1 $\pm$ 0	< 0.001	YES
Osx mRNA levels at day 14	4.99 $\pm$ 1.55	1.04 $\pm$ 0.56	< 0.001	YES
Osm mRNA levels at day 7	1.54 $\pm$ 0.2	1 $\pm$ 0	0.007	YES
Osm mRNA levels at day 14	1.91 $\pm$ 0.99	1.59 $\pm$ 0.47	0.568	No
Osn mRNA levels at day 7	1.27 $\pm$ 0.18	1 $\pm$ 0	0.048	YES
Osn mRNA levels at day 14	0.67 $\pm$ 0.45	1.3 $\pm$ 0.41	0.05	YES

**SYNOVIUM**

Synovium OI vs Synovium control

	SYN OI Mean $\pm$ SEM	SYN Control Mean $\pm$ SEM	P-VALUE	SIGNIFICANCE P-VALUE <0.05
ALP bio-activity at day 7	0.07 $\pm$ 0.01	0.05 $\pm$ 0.01	0.95	No
ALP bio-activity at Day 14	0.10 $\pm$ 0.02	0.03 $\pm$ 0.01	0.84	No
ALP mRNA levels at day 7	0.59 $\pm$ 0.21	0.31 $\pm$ 0.17	0.96	No
ALP mRNA levels at day 14	3.15 $\pm$ 1.07	0.75 $\pm$ 0.59	0.81	No
RUNX2 mRNA levels at day 7	0.80 $\pm$ 0.38	0.33 $\pm$ 0.15	0.53	No
RUNX2 mRNA levels at day 14	1.08 $\pm$ 0.46	0.82 $\pm$ 0.26	0.82	No
Osx mRNA levels at day 7	0.35 $\pm$ 0.34	0.02 $\pm$ 0.01	0.76	No
Osx mRNA levels at day 14	0.05 $\pm$ 0.03	0.04 $\pm$ 0.03	0.99	No
Osm mRNA levels at day 7	1.31 $\pm$ 0.69	0.4 $\pm$ 0.16	0.11	No
Osm mRNA levels at day 14	0.75 $\pm$ 0.41	0.36 $\pm$ 0.24	0.48	No
Osn mRNA levels at day 7	0.74 $\pm$ 0.17	0.82 $\pm$ 0.23	0.79	No
Osn mRNA levels at day 14	0.41 $\pm$ 0.15	1.14 $\pm$ 0.37	0.02	YES

**Table 3 (cont.)**ADIPOSE TISSUE

Adipose OI vs Adipose control

	AD OI Mean $\pm$ SEM	AD Control Mean $\pm$ SEM	P- VALUE	SIGNIFICANCE P-VALUE <0.05
ALP bio-activity at day 7	0.33 $\pm$ 0.13	0.07 $\pm$ 0.01	0.48	No
ALP bio-activity at Day 14	1 $\pm$ 0.34	0.06 $\pm$ 0.01	0.01	YES
ALP mRNA levels at day 7	3.37 $\pm$ 1.87	0.7 $\pm$ 0.63	0.92	No
ALP mRNA levels at day 14	5.52 $\pm$ 2.34	0.45 $\pm$ 0.42	0.68	No
RUNX2 mRNA levels at day 7	1.61 $\pm$ 0.89	0.86 $\pm$ 0.48	0.51	No
RUNX2 mRNA levels at day 14	0.76 $\pm$ 0.33	0.31 $\pm$ 0.14	0.69	No
Osx mRNA levels at day 7	0.06 $\pm$ 0.05	0.11 $\pm$ 0.10	0.96	No
Osx mRNA levels at day 14	0.18 $\pm$ 0.15	0.02 $\pm$ 0.01	0.87	No
Osm mRNA levels at day 7	0.61 $\pm$ 0.3	0.26 $\pm$ 0.14	0.53	No
Osm mRNA levels at day 14	0.13 $\pm$ 0.07	0.48 $\pm$ 0.38	0.54	No
Osn mRNA levels at day 7	0.36 $\pm$ 0.1	0.39 $\pm$ 0.19	0.91	No
Osn mRNA levels at day 14	0.14 $\pm$ 0.06	0.5 $\pm$ 0.14	0.26	No

**Table 4:** Comparative osteogenic capacities of BM-, SYN- and AD- MSCs

Overall effect of cell type

	P-VALUE	SIGNIFICANCE P-VALUE <0.05
ALP bio-activity	0.008	YES
ALP mRNA	0.02	YES
RUNX2	0.005	YES
OSTERIX	0.001	YES
OSTEOMODULIN	0.03	YES
OSTEONECTIN	0.02	YES

POST HOC TEST - HOLM SIDAK TEST

	SYN vs BM	FAT vs BM	SYN vs FAT
ALP bio-activity at day 7	YES 0.02	No 0.07	No 0.63
ALP bio-activity at Day 14	YES <0.001	YES 0.009	NO 0.11
ALP mRNA levels at day 7	YES 0.03	No 0.09	NO 0.16
ALP mRNA levels at day 14	YES 0.009	YES 0.01	No 0.86
RUNX2 mRNA levels at day 7	YES 0.007	YES 0.03	No 0.77
RUNX2 mRNA levels at day 14	YES 0.009	YES 0.006	No 0.83
Osx mRNA levels at day 7	YES 0.001	YES 0.001	No 0.84
Osx mRNA levels at day 14	YES 0.002	YES 0.002	No 0.92
Osm mRNA levels at day 7	No 0.09	YES 0.02	No 0.3
Osm mRNA levels at day 14	No 0.11	YES 0.02	No 0.39
Osn mRNA levels at day 7	YES 0.01	YES 0.002	No 0.23
Osn mRNA levels at day 14	No 0.4	No 0.1	No 0.3

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